

ONCOLOGIC DRUG ADVISORY COMMITTEE MEETING

HERCEPTIN[®] (Trastuzumab)

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HERCEPTIN® (Trastuzumab)

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EXECUTIVE SUMMARY

The primary purpose of this supplemental BLA is to seek regulatory approval for an alternative HER2 testing methodology, fluorescence in situ hybridization (FISH), to aid in the selection of patients for Herceptin[®] (Trastuzumab) therapy. The data presented address a post-approval commitment to “assess the clinical outcome of patients selected for treatment on the basis of the DAKO test [HercepTest[®]] and other HER2 diagnostics in the context of Herceptin clinical trials.”

Herceptin, a humanized anti-HER2 monoclonal antibody, received FDA approval in September 1998 for the treatment of HER2-overexpressing metastatic breast cancer, either in combination with paclitaxel for first-line therapy or as a single agent for second- or third-line therapy. This approval was based on two pivotal trials, Studies H0648g and H0649g. Eligibility for these pivotal trials, and for all Genentech-sponsored Phase II trials conducted prior to approval, required evidence of HER2 protein overexpression as determined by an immunohistochemistry (IHC) assay known as the Clinical Trials Assay (CTA).

Although the CTA was the method by which patients were selected for inclusion in the pivotal trials of Herceptin, it was impractical for commercialization and widespread clinical use. To provide clinicians with a method to select patients for Herceptin therapy, Genentech collaborated with a diagnostics company who developed an IHC assay system known as HercepTest[®] (DAKO, Inc., Carpinteria, CA). When HercepTest[®] was compared with the CTA in a sample of 548 breast tumor specimens, the agreement (concordance) between the two tests was 79%. This level of concordance is consistent with that seen in most concordance evaluations used for approval of IHC assays. The interpretation of this concordance analysis is contained in the current Herceptin label.

HercepTest[®] was the only diagnostic assay with a labeled indication to aid in the selection of patients likely to benefit from Herceptin therapy until November 2000. At that time, a second IHC assay (Pathway[™]; Ventana Medical Systems, Inc., Tuscon, AZ) was approved for this indication. Approval of the Pathway[™] assay was based on a concordance analysis between HercepTest[®] and Pathway[™]. These two IHC assays remain the only FDA-approved diagnostics to aid in the selection of patients for Herceptin therapy.

The mechanism of HER2 protein overexpression in human breast cancer involves amplification of the HER2 gene. Therefore, an alternative approach to identify patients for Herceptin therapy involves a direct assessment of HER2 gene copy number using FISH technology.

There are currently two FDA-approved HER2 FISH assay kits available commercially (PathVysion™, Vysis, Inc., Downer's Grove, IL, and INFORM™, Ventana Medical Systems, Inc.). These kits were approved based on their ability 1) to identify a population of node-positive breast cancer patients likely to benefit from anthracycline-based adjuvant therapy (PathVysion™) and 2) to identify a population of node-negative breast cancer patients with an adverse prognosis (INFORM™). It should be noted that these tests have not been approved for selection of patients for Herceptin therapy. Genentech, in conjunction with Vysis, is now presenting data that support the use of PathVysion™ to aid in the selection of patients likely to benefit from Herceptin therapy.

In order to evaluate FISH as a method to select patients for Herceptin therapy, Genentech conducted two separate assessments of this technology using PathVysion™. The first assessment was based on the same approach used by HercepTest® and Pathway™. This prospectively designed analysis assessed the concordance of HER2 protein overexpression by the CTA and HER2 gene amplification by FISH. The results of this analysis demonstrated an 82% overall concordance between the CTA and PathVysion™, consistent with the 79% concordance previously demonstrated between the CTA and HercepTest®. The second analysis involved a retrospective, exploratory assessment of FISH status as a predictor for clinical benefit (response rate, time to disease progression, and survival) from Herceptin therapy in three clinical trials, representing 799 patients. Results of these analyses from the two pivotal trials, representing 691 patients, are presented in this submission. These analyses support the use of FISH as an appropriate method to aid in the selection of patients for Herceptin therapy.

The concordance studies leading to FDA approval of HER2 diagnostics have been performed in a limited number of expert laboratories. These studies have consistently produced concordance in the 80%–90% range. Published reports suggest that this concordance can deteriorate as these assays are adopted by smaller, less-skilled, low-volume laboratories. Lack of adherence to the specific assay instructions, including specified reagents, precise procedures, and

consistent inclusion of controls for assay validation, is a common cause of errant results. Clinicians need to be alert to the issues associated with HER2 testing, regardless of methodology, and consider the results within the clinical context of the individual patient. Repeat testing using the same methodology or an alternative methodology may be required.

To date, all of the assays approved for selection of patients appropriate for Herceptin therapy have been based on concordance analyses. Ideally, assay selection would be based on patient outcome data. Genentech has considered prospective clinical studies to rigorously assess FISH as a method to select patients for Herceptin therapy. Studies randomizing patients to standard therapy with or without Herceptin would provide the most direct, conclusive, and reliable assessment. Because of the survival advantage demonstrated with Herceptin therapy, these studies in the metastatic breast cancer population would be unethical. However, in early stage breast cancer, the risk/benefit ratio of Herceptin therapy has not been established. Several large, ongoing adjuvant breast cancer studies in which patients are randomly assigned to receive treatment with or without Herceptin may provide, via a meta-analysis, adequate patient numbers to potentially isolate discordant clinical subsets (IHC+/FISH– and IHC–/FISH+) and to provide a reliable estimate of the clinical benefit of Herceptin in these subsets. It should also be recognized that other novel HER2 diagnostics are being developed, and trials to definitively evaluate each new assay methodology may be problematic.

Genentech believes that the data contained in this submission support changes to the Herceptin product labeling that include information regarding PathVysion™ as an alternative method of patient selection.

1. AN OVERVIEW OF HER2 BIOLOGY AND HERCEPTIN IN BREAST CANCER

The ErbB or HER family of receptor tyrosine kinases constitute a rich network of signaling pathways that culminate in cell growth, survival, and differentiation (for recent reviews, see Yarden and Sliwkowski 2001). Three of these receptors, EGFR, HER3, and HER4, bind, with somewhat redundant specificity, to 11 different gene products from the EGF-like superfamily. In normal biology, HER2 functions as a critical co-receptor dimerizing with other HER proteins to increase and/or initiate receptor–ligand signaling. When the HER2 gene is amplified in cancer, high levels of expressed HER2 protein form homodimers, resulting in constitutive, ligand-independent receptor activation.

Laboratory studies have demonstrated that HER2 transfection transforms normal cells and increases the aggressiveness of malignant cell lines (Di Fiore et al. 1987; Hudziak et al. 1987; Chazin et al. 1992; Pietras et al. 1995; D'souza and Taylor-Papadimitriou 1994). Additionally, transgenic female mice that are engineered to overexpress the gene in their breast tissue develop breast tumors when they reach reproductive age (Guy et al. 1992).

The clinical relevance of HER2 was first appreciated in 1987, when Slamon and co-workers identified a subset of breast cancer patients whose tumors contained an amplified version of the non-mutated HER2 gene (Slamon et al. 1987). These patients were noted to exhibit a more aggressive form of breast cancer (Slamon et al. 1989a). The details and significance of HER2 gene amplification and overexpression have been debated since this discovery. The general consensus is that HER2 overexpression does indeed occur in 20%–30% of all breast cancer patients (Ross and Fletcher 1998).

Several groups demonstrated that monoclonal antibodies directed against the activated forms of HER2 reverse many of the aggressive growth properties of HER2-transfected tumor cell lines (Drebin et al. 1985; Hudziak et al. 1989; Hancock et al. 1991; Stancovski et al. 1991; Harwerth et al. 1992; Kasprzyk et al. 1992). A panel of monoclonal antibodies was

generated at Genentech (Fendly et al. 1990); one of these antibodies, 4D5, was humanized to produce Herceptin[®] (Trastuzumab; see Appendix A for the current Herceptin[®] Package Insert) (Carter et al. 1992).

Herceptin is thought to inhibit the growth of breast cancer by at least two mechanisms (Sliwkowski et al. 1999). The first mechanism of action is down-regulation of constitutively activated receptors from the cell surface, resulting in a G0/G1 arrest. Growth arrest is preceded by induction of the CDK2 kinase inhibitor, p27^{KIP1}, and the retinoblastoma-related protein p130. A second mechanism of action for Herceptin involves the recruitment of immune effector cells to the tumor site through the interaction of the Fc region of Herceptin.

Preclinical evidence suggested that both the biochemical and the immunologic mechanisms of action are dependent on the level of HER2 expressed on the tumor cell surface. Tumors that express low or normal levels of HER2 were thought unlikely to benefit from Herceptin therapy. This led to a decision to limit enrollment in all Phase II and III Herceptin development trials to patients who demonstrated HER2 protein overexpression. Preliminary clinical data from a small group of HER2-negative patients treated with Herceptin appear to confirm this observation (Seidman et al. 2001).

2. ASSESSMENT OF HER2 STATUS

The prognostic value of a breast cancer patient's HER2 status has been debated since the initial report in 1987 (Slamon et al. 1987). The controversy that surrounds this topic can be traced to two principal causes. The first concerns variability in study design and conduct; two variables that greatly influence interpretation are insufficient cohort size and insufficient clinical follow-up (Ross and Fletcher 1998). The second cause is variability in specimen handling and the methodology of HER2 detection.

2.1 SOLID MATRIX BLOTTING TECHNIQUES

Solid matrix blotting procedures are well-established experimental techniques in any molecular biology laboratory. Methodology exists for accurate measurement of DNA, mRNA, and protein. In general, these techniques rely on extraction of the macromolecule of interest, separation by electrophoresis, transfer to a solid matrix (such as nitrocellulose), and detection with a specific probe followed by quantification. Variability in the methodology is corrected by normalization using a control macromolecule that is analyzed simultaneously with the sample. Selection of an inappropriate control may lead to an erroneous conclusion regarding the macromolecule of interest (Clark and McGuire 1991). Quantification of mRNA by Northern blot analysis from tumor specimens is difficult because of its intrinsic lability. Although proteins are generally more stable than mRNA, the preservation and analysis of any particular protein must be validated.

Southern, Northern, and Western blot procedures all share the common problem of variation in tumor content within a given specimen. Tumor tissue is composed not only of malignant cells but also of stromal, endothelial, and inflammatory cells, which may constitute more than 50% of any breast tumor specimen (Slamon et al. 1989b). Since these elements vary from tumor to tumor, dilutional artifacts may be introduced when analyzing a particular gene or its product. The emergence of immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) techniques has largely circumvented this flaw in solid matrix blotting techniques. An additional limitation is that solid matrix blotting techniques are best performed with fresh frozen tumor tissue. Since most clinical institutions do not currently archive their patients' breast tumor specimens by freezing, samples are generally not available for solid matrix blotting techniques.

2.2 COMPARISON OF HER2 GENE AMPLIFICATION AND PROTEIN OVEREXPRESSION IN HUMAN BREAST CANCER ARCHIVAL MATERIAL

In practical terms, the fact that fresh frozen tumor tissue is rarely available for most breast cancer patients limits the methodologies used to ascertain a patient's HER2 status. Two robust technologies, IHC and FISH, are now accepted methods for determining HER2 levels in tumor specimens that are readily available, namely fixed, paraffin-embedded tissue. An appealing feature of these techniques is that they assess HER2 protein expression and gene amplification on a cell-by-cell basis, thus eliminating the problem of dilutional artifacts.

2.2.1 Immunohistochemistry

a. General Considerations

IHC is a widely used method for detecting protein expression in tissue sections. Essentially, this method detects an epitope on a protein in a tissue section by using an antibody specific to that epitope. The reliability of the final result is dependent on the specificity of the primary antibody and on how successfully the target epitope is preserved and then detected in the patient sample.

The advantages of IHC include its wide availability, speed and simplicity in performance, and relative low expense. By using consistently preserved and processed tissue samples and standardized laboratory protocols, reliable IHC results, demonstrating good intra- and inter-laboratory agreement, can be obtained. The equipment and materials needed to perform IHC are present and used routinely in most pathology laboratories.

Most of the disadvantages of IHC result from the lack of standardization and control over all aspects of the procedure. Although IHC has been used in pathology laboratories for over 20 years, strict performance standards have not been established, resulting in extensive variability (Hilborne and Nathan 1991; Taylor 1993, 2000; Taylor and Cote 1994).

The College of American Pathologists has recently published guidelines with the goal of achieving standardization in the performance of IHC (Fitzgibbons et al. 2000).

There are numerous technical issues that can affect the outcome of an IHC assay. These include the type and extent of tissue fixation, whether antigen retrieval is employed, the antibody used, sensitivity of the visualization method, and standardization of scoring (see Table 1 for examples of technical issues affecting IHC outcomes).

Table 1
Technical Issues Affecting IHC Outcomes

Elements of Test Process	Variable Affecting Outcome	Potential Impact	Potential Outcome	Reference
Fixation	Choice of fixative: many are commercially available and in wide use. Some have been developed specifically to preserve epitope, which increases sensitivity of IHC. (HercepTest [®] specifies 10% neutral buffered formalin or Bouin's fixed tissue.)	Some fixatives developed specifically for IHC (e.g., zinc formalin, alcohol-based fixatives) can increase IHC sensitivity.	False positive	Farnilo and Stead 1989; Penault-Llorca et al. 1994
	Time elapse from specimen collection to immersion in fixative.	Delay in fixation may cause permanent loss of protein through degradation.	False negative	
	Duration of fixation.	Under-fixation: permanent loss of protein.	False negative	
		Over-fixation: increased protein crosslinking may cause decreased availability of epitope for detection.	False negative	
	Volume of fixative too small or portion of tissue too large for fixative volume.	Under-fixation: permanent loss of protein.	False negative	
Antibody	Many anti-HER2 antibodies are commercially available. Two FDA-approved methods.	Variability in sensitivity of antibodies.	False negative; false positive	Press et al. 1994; Birner et al. 2001
Antigen retrieval (syn: epitope retrieval, unmasking)	Many methods and procedures are available; primary categories are enzymatic and heat-induced. (HercepTest [®] specifies waterbath method of heat-induced epitope retrieval.)	Varying degrees of restoration or permanent destruction of epitopes.	False negative; false positives	Battifora and Kopinski 1986; Shi et al. 2001
Interpretation	Experience and basis of scoring criteria (HercepTest [®] specifies a 0 to 3+ scale adapted from the Herceptin CTA scoring system.)	Varies from pathologist to pathologist.	False negative; false positive	Jacobs et al. 1999b ; Hammond et al. 2000

b. Use of IHC to Assess HER2 Protein Expression

IHC provides a readily available method of assessing HER2 protein expression; however, accurate detection of HER2 overexpression using IHC presents a significant challenge. HER2 overexpression must be detected against the background of normal HER2 expression present in breast tissue (Press et al. 1990). Therefore, the technical issues that can influence IHC outcomes are magnified in their potential to affect the results of HER2 assessments in particular.

The FDA-approved HercepTest[®] was developed to minimize variability by standardizing the technical aspects of IHC; however, even with the availability of this standardized IHC kit, wide variation in results continues to be reported (Jacobs et al. 1999a, 1999b; Tubbs et al. 2001). A second IHC reagent (Pathway[™] HER2 for use with the BenchMark[™] automated system; Ventana Medical Systems, Inc., Tuscon, AZ) has now been approved as an aid in the selection of patients likely to benefit from Herceptin therapy. This approval was based on a concordance analysis between HercepTest[®] and Pathway[™] (see Table 3 of Appendix B). These two IHC assays remain the only FDA-approved diagnostics to aid in the selection of patients for Herceptin therapy.

2.2.2 Fluorescence In Situ Hybridization

a. General Considerations

FISH is a DNA-based methodology that allows for the detection of gene copy number within cells in tissue sections. DNA is inherently more stable than protein because it is less subject to biochemical degradation, resulting in a more durable target in patient samples. As a result, the availability of more consistent patient samples and the use of standardized assay protocols provide the ability to achieve consistent and reliable results with FISH.

FISH detects a specific sequence of DNA within a gene in a tissue section by using a labeled complementary DNA probe. The reliability of the final result is dependent on the specificity of the probe and how successfully

the target portion of the gene of interest is exposed in the patient sample. There are a variety of DNA probes of different sizes, including locus-specific probes (which target unique DNA sequences) and alpha satellite probes (which target centromeric repeat sequences). Both direct and indirect methods of detection can be employed.

The current disadvantages of FISH are related in part to the relative inexperience of the pathology community with this technique. Although the equipment and materials needed to perform FISH are present and used routinely in medical genetics laboratories, they are not yet widely available in pathology laboratories. Technical issues can affect the outcome of a FISH assay. These include the type of tissue fixation, whether a directly or indirectly labeled probe is used, and selection of the correct (invasive tumor) area for scoring (see Table 2 for examples of technical issues affecting FISH outcomes).

Table 2
Technical Issues Affecting FISH Outcomes

Elements of Test Process	Variable Affecting Outcome	Potential Impact	Potential Outcome	Reference
Fixation	Choice of fixative: many are commercially available and in wide use. (PathVysion™ specifies 10% neutral buffered formalin.) pH: fixatives range from alkaline to acidic. Duration of fixation	Over-fixation: excessive protein crosslinking may increase difficulty of removing protein and crosslinks from DNA, resulting in decreased probe penetration.	False negative	Fletcher 1999; Basyuk et al. 2000
DNA unmasking	Enzymatic digestion step required to remove protein, which forms a barrier between probes and chromosomal domains to which they hybridize. (PathVysion™ includes pepsin pretreatment step.)	Restoration of access to DNA is dependent on fixative used and duration of fixation for a given tissue. Under-digestion: barrier to probes may remain. Over-digestion: potential loss of nuclear architecture and chromosome integrity.	False negative Assay failure	Fletcher 1999;
Interpretation	Selection of area to score may be difficult because of dark-field visualization with FISH, e.g., must discriminate between invasive carcinoma and DCIS or normal (stromal) and malignant cells.	Non-representative scoring.	False positive; false negative	Fletcher 1999
Microscopy	High-quality epi-illumination fluorescence microscope. (PathVysion™-specific filter sets for excitation of SpectrumOrange and SpectrumGreen recommended.)	Poor quality optics or substitute filter sets may lead to underscoring.	False negative	Dewald et al. 1998

DCIS = ductal carcinoma in situ.

b. Use of FISH to Measure HER2 Gene Copy Number

Although FISH does not measure HER2 protein expression directly, a number of studies have addressed this issue and have observed >95% correlation between gene amplification and protein overexpression when evaluated in frozen tissues not compromised by fixation (Pauletti et al. 1996; Slamon et al. 1989a; Naber et al. 1990; Kallioniemi et al. 1992). In these studies, gene amplification without associated protein overexpression was never observed. Protein overexpression without gene amplification (single-copy overexpression) is rare (~2% of HER2-positive breast cancer). Therefore, direct assessment of HER2 gene copy number using FISH offers an alternative method for determining HER2 overexpression.

There are currently two FDA-approved HER2 FISH assay kits available commercially (PathVysion™, Vysis, Inc. and INFORM™, Ventana Medical Systems, Inc.; see Appendix C for the PathVysion™ Package Insert). These kits were approved based on their ability 1) to identify a population of node-positive breast cancer patients specifically benefiting from anthracycline-based adjuvant therapy (PathVysion™) and 2) to identify a population of node-negative breast cancer patients with an adverse prognosis (INFORM™).

The PathVysion™ kit includes two directly labeled probes: a locus-specific HER2 probe and an alpha satellite probe targeting the centromere region of chromosome 17 (CEP17). By enumerating copy numbers of HER2 and CEP17 in dual colors, the ratio of the average copy numbers per cell can be calculated to determine the presence of amplified HER2. A ratio of ≥ 2 has been established as an optimal cut-point to differentiate amplified versus non-amplified samples. The CEP17 probe provides an internal hybridization control, minimizing the likelihood of assay failure and a false-negative result and correcting for possible aneusomy involving chromosome 17. The INFORM™ kit includes a single biotin-labeled locus-specific HER2 probe, which is detected indirectly. HER2 gene copy number is enumerated without normalizing for chromosome 17 copy number since the INFORM™ kit does not include a centromeric control

probe. As a result, a HER2 gene copy number of ≥ 4 has been established as the optimum cut-point to differentiate amplified versus non-amplified samples.

Although the technology of FISH was initially described in 1986 and the first report of its use for HER2 detection appeared in 1992, Genentech elected to use IHC as the methodology to select patients for inclusion in the Phase II and pivotal clinical trials. This decision was based on the widespread availability and general acceptance of IHC technology by pathologists, as well as the efforts carried out to validate the CTA.

Since that time, two FISH assays for HER2 detection have been developed and approved for clinical use. Genentech sought to evaluate this technology to aid in the selection of patients for Herceptin therapy by conducting two separate studies. The first involved a prospectively designed concordance analysis between HER2 protein overexpression by the CTA and HER2 gene amplification by FISH. The second involved a retrospective, exploratory assessment of FISH status as a predictor of clinical benefit from Herceptin in three clinical trials representing 799 patients. Results of these analyses from the two pivotal trials, representing 691 patients, are presented in this submission. Each of these studies was conducted using PathVysion™. This system was selected based on the theoretic advantages of the inclusion of a centromeric control probe to correct for the possibility of aneusomy involving chromosome 17 and the enhanced assay reliability provided by direct fluorescence labeling.

3. CONCORDANCE ANALYSIS

3.1 GOALS AND OBJECTIVES

The concordance study was prospectively designed to establish the relationship between the CTA (the IHC assay used to identify eligible patients for the Herceptin pivotal trials) and HER2 gene amplification as determined by FISH. The analysis involved estimating the overall 2×2 concordance, the extrapolated concordance within the population of metastatic breast cancer patients screened by the CTA, and the rates of

HER2 gene amplification within each CTA score. IHC assays for HER2 detection (e.g., HercepTest[®] and Pathway[™]; see Appendix B) have received FDA approval based on establishing acceptable concordance between these assays and the CTA and HercepTest[®], respectively.

3.2 STATISTICAL PLAN

Genentech elected to replicate the statistical plan used for the original HercepTest[®]/CTA concordance study, which was conducted and included in the original Herceptin BLA. The concordance level between the IHC and FISH assays was determined as the proportion of samples rated either positive or negative by both assays in a sample with an equal proportion of CTA-positive (2+, 3+) and CTA-negative (0, 1+) tissues. FISH+ was defined as a signal ratio of ≥ 2 . To determine whether the concordance level was acceptable, a one-sided test was conducted on the concordance rate at the 5% significance level. Assuming that a concordance level of $\leq 75\%$ reflects disagreement between the assays, a sample size of approximately 600 was required in order to provide 90% power to detect a level that was 5% higher than the unacceptable level of concordance.

In the secondary analysis, κ statistics were evaluated. The κ statistic is a measure of inter-assay agreement. Values for the κ statistic between 0.4 and 0.75 represent fair to good agreement beyond chance, and values above 0.75 represent excellent agreement beyond chance (Fleiss and Joseph 1981).

3.3 SPECIMEN IDENTIFICATION

A total of 5998 patients had tumor samples submitted to a single reference laboratory (Laboratory Corporation of America, "LabCorp") between 1994 and 1997. These samples had been submitted specifically for assessment of HER2 status using the CTA in order to determine eligibility for several Herceptin clinical trials. Of these 5998 patients, 1915 (32%) were scored as 2+ or 3+ ("positive") and 4083 (68%) were scored as 0 or 1+ ("negative"). Study sites were instructed to provide representative

tumor blocks to LabCorp, from which nine tissue sections were typically cut, and the blocks were returned to the sites. In situations in which the study site was unwilling or unable to provide a tumor block, eight to ten slides (sometimes fewer) of 4- to 6- μ m tissue sections were sent directly to LabCorp. The CTA required the use of at least four tissue sections, and frequently additional sections were needed for repeat assays due to technical issues. For patients subsequently enrolled in Herceptin clinical trials, confirmation of estrogen and progesterone receptor status was also carried out, consuming additional tissue sections. Therefore, not all patients had additional unstained tissue sections. From the total population of 5998 CTA-screened patients, 5251 (87%) had at least two unused tissue sections that had been archived at LabCorp for 2 to 5 years and were available for FISH analysis. This was the study population used for the concordance analysis. The IHC distribution was similar among the total 5998 patients, the 5251 constituting the study population, and the 747 patients without additional slides.

3.4 METHODOLOGY

From the population of 5251 patient specimens, Genentech generated a random sample of 623 specimens, with a 1:1 ratio of HER2-positive and HER2-negative samples: 317 CTA-positive (2+/3+) and 306 CTA-negative (0/1+) specimens. This list was provided to personnel at LabCorp, who located the specimens and re-assigned a new, unique sample identification number in order to accomplish blinding to the prior CTA results at LabCorp.

FISH assays were performed using PathVysion™ with minor modifications required by the unique condition of the samples analyzed:

- Minor changes to the pretreatment and protease digestion times were made based on the age and condition of the specimens. These variations were instituted after consultation with the Vysis Technical Department and were within the technical variations permitted by the PathVysion™ package labeling (see Appendix C).
- For tissue sections that were too large to be adequately covered by a standard 22 × 22 mm coverslip, a 24 × 40 mm coverslip was used. An

assessment of the effect of probe volume and coverslip size is contained in Appendix D.

- For samples that were clearly non-amplified (signal ratio of <1.8) or clearly amplified (signal ratio of >2.2) after 40 cells were enumerated, no further counting was performed. If the ratio fell between 1.8 and 2.2, an additional 20 cells were enumerated, for a total of 60. An assessment of the effect of 40 versus 60 nuclei enumeration is contained in Appendix E.

3.5 RESULTS

A total of 317 CTA-positive specimens and 306 CTA-negative specimens were identified and tested with PathVysion™. From these 623 samples, an informative FISH result was obtained in 529 (85%) cases. The non-informative cases were typically due to an inability to discretely separate and confidently enumerate signals. These non-informative results were likely the result of a standardized protease digestion step, which may have been suboptimal for some clinical samples.

The overall 4 × 2 concordance table, based on the 529 samples with informative results, is shown in Table 3. The 2 × 2 concordance was defined as the proportion of samples rated 0 or 1+ by the CTA and not amplified by FISH plus the proportion of samples rated 2+ or 3+ by the CTA and amplified by FISH. The amplification rates by CTA scores are shown in Table 4.

Table 3
FISH/CTA Concordance

Gene Amplification by FISH	CTA Score				Total
	0	1+	2+	3+	
No	207	28	67	21	323
Yes	7	2	21	176	196
Total	214	30	88	197	529
Distribution (%)	40	6	17	37	100

Note: Concordance, 82% (95% CI of 78%, 85%). κ statistic: 0.64 (95% CI of 0.58, 0.7).

Table 4
Amplification Rate by CTA Score

	CTA Score			
	0	1+	2+	3+
Percent amplified	3%	7%	24%	89%

Gene amplification was defined as a HER2:CEP17 signal ratio of ≥ 2 (consistent with the PathVysion™ Package Insert).

3.6 INTERPRETATION AND CONCLUSIONS

a. Concordance

The analysis of concordance is maximally informative if approximately equal numbers of positive and negative cases are included. It is important to note that this study was designed to include an approximately equal number of CTA-positive and CTA-negative cases. The 2×2 overall sample concordance was 82%, which is consistent with the 79% concordance demonstrated between the CTA and HercepTest® (see Appendix B). The κ statistic is 0.64, indicating good agreement. This suggests that using PathVysion™ to aid in the selection of patients for Herceptin therapy should provide information similar to that currently provided by HercepTest®.

b. Extrapolated Concordance

Table 5 reflects the extrapolated concordance that would be expected in the population of metastatic breast cancer patients screened using the CTA.

Table 5
Extrapolated Concordance

Gene Amplification by FISH	CTA Score				Total
	0	1+	2+	3+	
No	298	44	40	13	395
Yes	9	3	13	109	134
Total	307	47	53	122	529
Distribution (%)	58	9	10	23	100

Note: Extrapolated concordance, 88% (95% CI of 85%, 91%).

The extrapolated concordance is 88%, which is consistent with the extrapolated concordance noted between HercepTest[®] and the CTA (83%; see Appendix B). Based on this analysis, one can estimate the correlation of the PathVysion[™] results with the CTA results. Of specimens testing FISH+ by PathVysion[™], 91% would be expected to test at least 2+ in the CTA (i.e., meeting the Herceptin study entry criterion), including 81% that would be expected to test 3+, and 9% that would be expected to score 0 or 1+.

4. CLINICAL OUTCOMES

4.1 GOALS AND OBJECTIVES

After establishing concordance between the CTA and HER2 gene amplification as measured by FISH, Genentech sought to explore the relationship between HER2 gene amplification status (FISH+ and FISH-) and Herceptin clinical benefit in three Genentech-sponsored clinical trials of Herceptin: Studies H0648g, H0649g (the two pivotal trials supporting the initial BLA submission), and H0650g (a Phase II trial evaluating two dose levels of Herceptin monotherapy in previously untreated patients with HER2-overexpressing metastatic breast cancer). Only clinical outcome data from the two pivotal trials are presented in this submission.

4.2 STUDY POPULATION

The study population included the 799 patients treated in the three clinical trials: Studies H0648g, H0649g, and H0650g. A total of 805 patients were treated in these three studies, although 6 patients initially enrolled in Study H0650g were subsequently enrolled in Study H0648g.

4.3 STUDY DESCRIPTIONS

Table 6 summarizes the three clinical trials—H0649g, H0648g, and H0650g—that contributed data to the clinical outcomes analysis and laboratory validation analyses (see Section 4.6.3).

Table 6
Table of Studies

Study	Study Title	Efficacy Endpoints	Dosing Regimen	Patients Enrolled
H0649g (pivotal)	A Multinational, Open-Label Study of Recombinant Humanized Anti-p185 ^{HER2} Monoclonal Antibody (rhuMAb HER2) in Patients with HER2/ <i>neu</i> Overexpression Who Have Relapsed following One or Two Cytotoxic Chemotherapy Regimens for Metastatic Breast Cancer	<ul style="list-style-type: none"> • Overall response rate (primary) • Duration of response • Time to disease progression • Time to treatment failure • Survival time 	Multiple dose: weekly 4 mg/kg LD/ 2 mg/kg MD	222
H0648g (pivotal, Phase III)	Chemotherapy and Antibody Response Evaluation (CARE): a Phase III, Multinational, Randomized Study of Recombinant Humanized Anti-p185 ^{HER2} Monoclonal Antibody (rhuMAb HER2) Combined with Chemotherapy in Patients with HER2 Overexpression Who Have Not Received Cytotoxic Chemotherapy for Metastatic Breast Cancer	<ul style="list-style-type: none"> • Time to disease progression (primary) • Overall response rates • Duration of major response • Time to treatment failure • Survival time 	Multiple dose: weekly 4 mg/kg LD/ 2 mg/kg MD (chemotherapy alone or Herceptin + chemotherapy)	469
H0650g ^a (Phase II)	A Multinational, Randomized, Single-Blind Study of Recombinant Humanized Anti-p185 ^{HER2} Monoclonal Antibody (rhuMAb HER2) in Patients with HER2/ <i>neu</i> Overexpression Who Have Not Received Prior Cytotoxic Chemotherapy for Metastatic Breast Cancer	<ul style="list-style-type: none"> • Overall response rate (primary) • Time to disease progression • Response duration • Survival time 	Multiple dose: weekly 4 mg/kg LD/2 mg/kg MD or 8 mg/kg LD/ 4 mg/kg MD	114

LD = loading dose; MD = maintenance dose.

^a FISH results from Study H0650g were used only for the validation analyses.

4.3.1 Study H0649g

Study H0649g was an open-label, Phase II trial of Herceptin in patients with HER2 overexpression who had relapsed following one or two cytotoxic chemotherapy regimens for metastatic breast cancer.

The primary endpoint of the study was overall response rate as determined by an external, blinded Response Evaluation Committee (REC). Secondary efficacy endpoints included time to disease progression, duration of response, and survival (see Table 6).

A total of 222 patients were enrolled into Study H0649g. The data cutoff date was October 1999. In this study, when Herceptin was used as a single agent in patients who had previously received chemotherapy for metastatic disease, objective, durable responses were observed.

4.3.2 Study H0648g

Study H0648g was a Phase III, randomized, controlled trial of chemotherapy alone and in combination with Herceptin in patients with HER2-overexpressing tumors who had never received cytotoxic chemotherapy for metastatic breast cancer. Patients were randomized in a balanced fashion to one of two treatment groups: 1) Herceptin + chemotherapy or 2) chemotherapy alone (no Herceptin). The chemotherapy regimen for both treatment groups was either anthracycline + cyclophosphamide (AC) or paclitaxel. Patients who had not received anthracycline therapy in the adjuvant setting were stratified to receive AC. Patients who had received any anthracycline therapy in the adjuvant setting were stratified to receive paclitaxel.

The primary endpoint of the study was time to disease progression as determined by an external, blinded REC. Secondary efficacy endpoints included overall response rates, duration of response, and survival (see Table 6).

A total of 469 patients were enrolled into Study H0648g. The data cutoff date was October 1999. In this study, when Herceptin was added to

standard chemotherapy as first-line treatment for metastatic disease, significant improvements were noted in time to disease progression, overall response rates, duration of response, and survival.

4.3.3 Study H0650g

Study H0650g was a randomized, single-blind trial of the efficacy and safety of Herceptin as a single agent in patients with HER2-overexpressing tumors who had never received and did not wish to receive cytotoxic chemotherapy for metastatic breast cancer. Patients were randomized to one of two regimens of Herceptin: 1) a 4 mg/kg loading dose on Day 0, followed by 2 mg/kg weekly until disease progression (the “standard” dose used in Studies H0648g and H0649g), or 2) an 8 mg/kg loading dose on Day 0, followed by 4 mg/kg weekly until disease progression.

The primary endpoint of the study was overall response rate. Secondary endpoints included time to disease progression, duration of response, and survival (see Table 6). The efficacy endpoints in this study were determined by the investigators, not by an external, blinded REC.

A total of 114 patients were enrolled into Study H0650g. The data cutoff date was October 1999. In this study, when Herceptin was used as a single agent in previously untreated patients with HER2-overexpressing metastatic breast cancer, meaningful, durable responses were observed.

For the purposes of this submission, FISH results obtained for samples from patients enrolled in Study H0650g were used only for laboratory validation analyses.

4.4 STATISTICAL ANALYSES

This analysis was an exploratory, retrospective assessment of FISH status as a predictor for clinical benefit in the aforementioned three Herceptin clinical trials (data from only the two pivotal trials are presented). A formal, prospectively defined statistical plan was not established. Instead, the prospectively designed statistical analysis plan for each of these trials

was used as a guide to ensure consistency of approach between the initial study analysis and the post hoc analysis. For the three trials, each patient was classified as FISH+ (signal ratio of ≥ 2) or FISH- (signal ratio of < 2). Then the primary and secondary analyses, including both efficacy and safety endpoints as defined in each of the original clinical trials, were performed based on the two newly defined (FISH+ and FISH-) subpopulations.

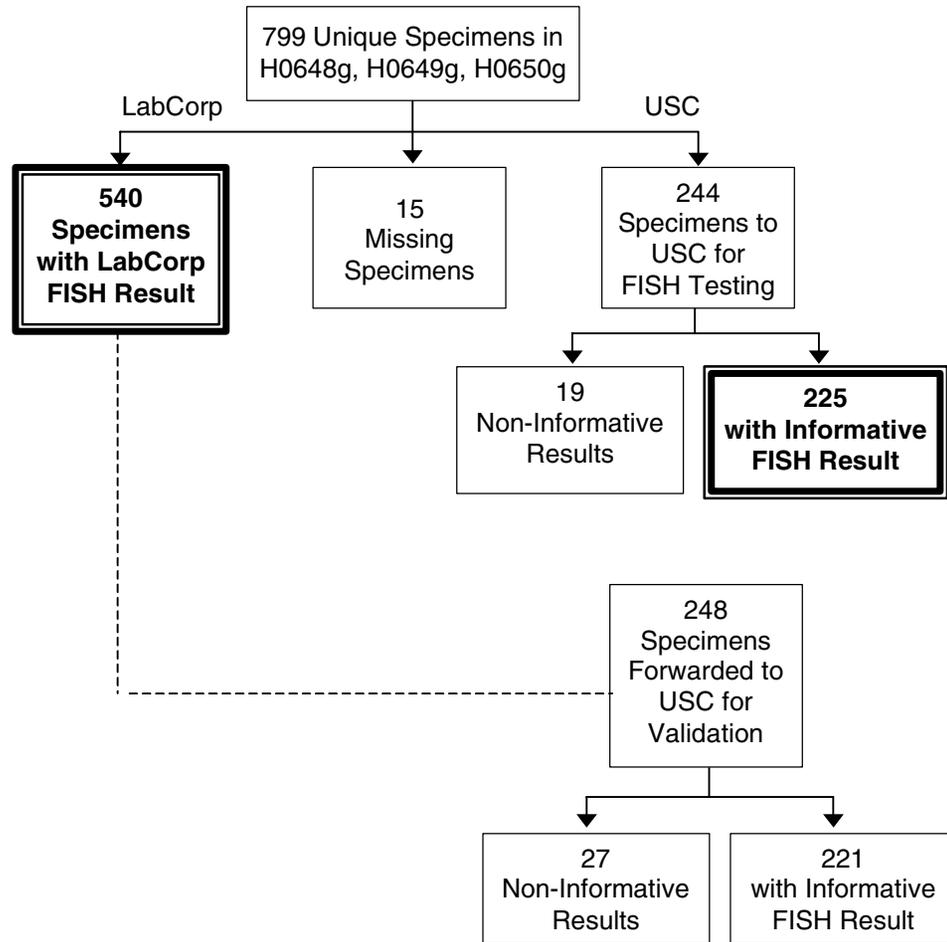
4.5 SPECIMEN IDENTIFICATION

Genentech initially identified the 799 patients enrolled in the three clinical trials. A total of 15 were enrolled based on evidence of HER2 overexpression as determined by HER2 gene amplification, using either solid matrix blotting or FISH performed locally. These patients did not have tissue submitted to, or archived at, LabCorp. Of the remaining 784 patients, 618 (79%) were found to have at least two additional unstained slides archived at LabCorp and available for FISH. The same specimen number re-assignment procedures used for the concordance analysis were applied to these samples in order to blind LabCorp personnel to the original CTA scores. From these samples, FISH results were obtained in 540 of 618 (87%) cases, with a corresponding non-informative rate of 13%.

In order to minimize the number of patients lacking a FISH result, Genentech elected to perform additional FISH assays in a separate laboratory (The University of Southern California, "USC") using tissue sections that had been previously immunostained. Specifically, these sections were the control IgG slides used either for the CTA or for the estrogen/progesterone receptor assays. Preliminary data had suggested that FISH results could be generated in these tissue sections. A total of 244 patient specimens were forwarded to USC for FISH testing, and FISH results were obtained for 225 specimens. An additional 248 specimens with known FISH results from LabCorp were also forwarded to USC for validation.

Figure 1 presents the specimen flowchart.

Figure 1
Specimen Testing Flowchart



The data shown in Table 7 were obtained by combining FISH results from the two laboratories.

Table 7
Availability of FISH Results

Study	Patients with Available FISH Data
H0648g	451/469 (96.2%)
H0649g	209/222 (94.1%)
H0650g	111/114 (97.4%)
All individual patients ^a	765/799 (95.7%)

^a Six patients from Study H0650g were later enrolled into Study H0648g. FISH data are available for these 6 patients.

4.6 METHODS

4.6.1 LabCorp

The initial FISH assays on the 618 previously unstained tissue sections were performed at LabCorp on archived (non-coverslipped) tissue sections. They were processed in one of two methods: using either standard manual protease digestion or the automated VP2000 autostainer, which also employs a standardized protease digestion step. After processing (de-paraffinization and protease digestion), probe hybridization conditions and the signal enumeration protocol were identical. Only a single standardized attempt at protease digestion and hybridization was performed at LabCorp.

4.6.2 USC

For these samples, which were previously immunostained and coverslipped tissue sections, the following processing algorithm was used. The coverslip was first removed by prolonged (48–96 hours) soaking in xylene followed by rinsing in ethanol. Each tissue section was initially processed using the VP2000 autostainer, with standardized de-paraffinization and protease digestion. If hybridization and interpretation were successful, the resulting FISH score was recorded. If

hybridization and interpretation were unsuccessful, the tissue section was subjected to individualized protease digestion under microscopy. This technique optimized probe hybridization conditions. Repeated efforts to optimize the hybridization conditions were undertaken provided the tissue section remained intact or additional tissue sections were available. This individualized approach led to a lower non-informative assay rate at USC. Of the 244 samples, which included 78 (618 minus 540) that gave a non-informative result at LabCorp, results were obtained in 225 cases.

4.6.3 Validation Studies

The data shown in Table 7 represent pooling of FISH results from the two laboratories (LabCorp and USC). To validate pooling of the data, specimens with a known FISH result from LabCorp were forwarded for re-testing to USC in a sequential process.

Initially, 90 randomly selected patients, with an equal proportion of FISH+ and FISH- tissues, from the 540 with a known FISH result at LabCorp were identified and forwarded to USC. This number was selected assuming that the true inter-laboratory concordance was 95% and that the lower limit of the 95% confidence interval would exceed 85%. Results of this concordance analysis for the two laboratories are presented in Table 8.

Table 8
Results of Validation Analysis

		USC Result		Total ^a
		FISH+	FISH-	
LabCorp result	FISH+	37	2	39
	FISH-	15	28	43
	Total	52	30	82

^a Of the 90 requested cases, 1 had a missing slide and 7 yielded non-informative results (no invasive tumor present [4 cases]; over-digestion without an additional slide available [3 cases]).

This analysis demonstrated an overall concordance of 79%, which fell below the prespecified concordance of >85%. However, it appeared that

the discordance was restricted primarily to those cases scored as “negative” at LabCorp and subsequently scored as “positive” at USC (15 of 17 discordant cases).

When Genentech examined the IHC scores of those 15 discordant cases (LabCorp negative, USC positive), it was determined that 13 of 15 (87%) had been scored as 3+ by the CTA. Because the FISH/CTA concordance (see Section 3.5) had demonstrated 89% amplification in the 3+ group, this suggested that these 15 previously negative specimens (by LabCorp) might actually have been amplified, consistent with the USC determination. These data suggested that LabCorp might have systematically underscored these FISH assays.

To test our hypothesis that differences in tissue preparation and processing had led to systematic discordance between the two laboratories, the following steps were initiated:

- All (108) of the remaining specimens with a known “negative” FISH score at LabCorp were sent to USC for re-testing.
- An additional random sample of 50 specimens with a known “positive” FISH score at LabCorp was also sent to USC for re-testing.

The results of this additional re-testing of the 158 specimens at the USC laboratory are presented in Table 9.

Table 9

Results of Additional Validation Analyses

		USC Result		Total
		FISH+	FISH-	
LabCorp result	FISH+	42	0	42 ^a
	FISH-	22	75	97 ^b
	Total	64	75	139

^a Of the 50 samples determined to be FISH+ by LabCorp, 8 yielded non-informative results (only hematoxylin and eosin slides provided [2 cases], no invasive tumor present [1 case], specimen over-digestion [2 cases], high background [1 case], weak signal [1 case], tissue section fell off slide during processing [1 case]).

^b Of the 108 samples determined to be FISH- by LabCorp, 11 yielded non-informative results (only hematoxylin and eosin slides provided [1 case], no invasive tumor present [1 case], specimen over-digestion [3 cases], weak signal [6 cases]).

When these data were combined with the original validation results, the overall results shown in Table 10 were obtained. Although the overall concordance in these 221 samples was 82%, the following agreement between the laboratories was observed:

- Of the 81 FISH+ samples from LabCorp with a FISH result obtained at USC, 79 were FISH+ (agreement = 97.5%).
- Of the 140 FISH- samples from LabCorp with a FISH result obtained at USC, 103 were FISH- and 37 were FISH+ (agreement = 73.6%).

Table 10

Overall Validation Results

		USC Result		Total ^a
		FISH+	FISH-	
LabCorp result	FISH+	79	2	81
	FISH-	37	103	140
	Total	106	105	221

Note: Concordance, 82% (95% CI of 76%, 87%).

These results appear to confirm our hypothesis that systematic underscoring may have occurred at LabCorp. Positive FISH results were nearly always confirmed at USC. Differences in the specimens used

(archived, non-coverslipped, unstained tissue sections at LabCorp vs. archived, coverslipped, previously stained tissue sections at USC) may have contributed to the underscoring seen at LabCorp.

See Appendix F for a comparison of the methods used at LabCorp and USC.

4.7 RESULTS

For the reporting of results, Genentech defined the “primary” dataset as the original 540 results from LabCorp combined with the 225 additional results from USC (total n = 765/799 results). After completion of the validation process, Genentech also defined a “secondary” dataset that gave precedence to results from USC. For any patient with a FISH result from both laboratories, the USC result was used for the secondary dataset. If no result was available from USC, the LabCorp result was used. Tables and Kaplan-Meier plots for the secondary analysis are included in Appendix G.

4.7.1 Study H0649g

Table 11 shows the response rate for the two newly defined subpopulations (FISH+ and FISH–) in Study H0649g, the open-label, single-agent trial in HER2-overexpressing patients who had relapsed following one or two cytotoxic chemotherapy regimens for metastatic breast cancer. It is noteworthy that there were no objective responses among the 46 patients found to be FISH– as compared with a 20% response rate among the 163 patients noted to be FISH+.

Table 11

Overall Response Rate in Study H0649g for the Newly Defined Patient Subpopulations

FISH+	
n/N	33/163
%	20
95% CI	(14.4, 27.2)
FISH-	
n/N	0/46
%	0
95% CI	(0.0, 7.7)

Figures 2 and 3 show the Kaplan-Meier curves for time to disease progression and overall survival for the newly defined subpopulations.

Figure 2

Time to Disease Progression for FISH+ and FISH- Patients in Study H0649g

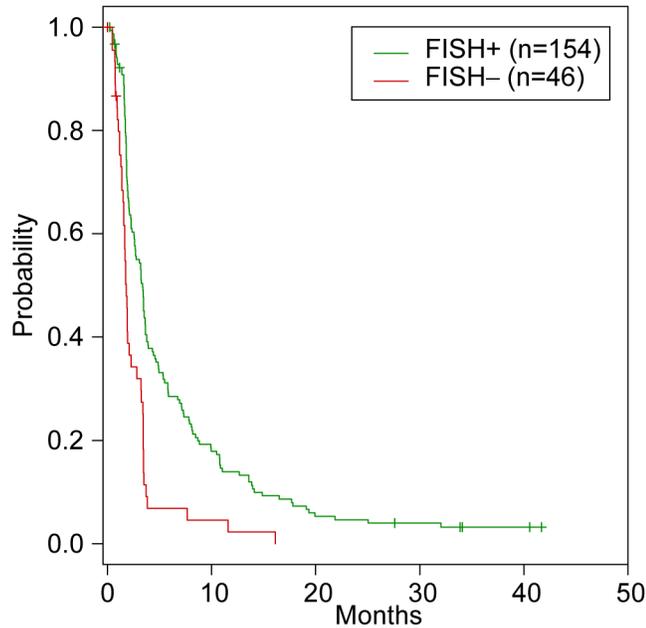


Figure 3

Survival Time for FISH+ and FISH- Patients in Study H0649g

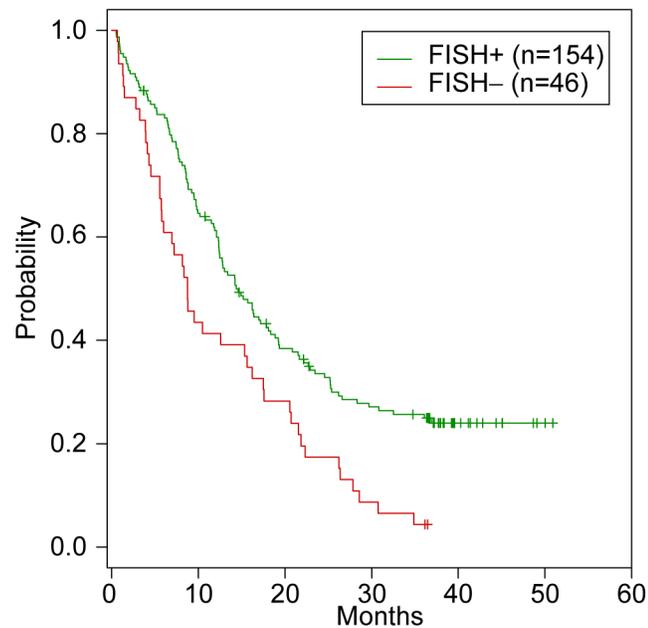
**4.7.2 Study H0648g**

Table 12 shows the response rate for the two newly defined subpopulations (FISH+ and FISH-) in Study H0648g, the Phase III, randomized trial of Herceptin combined with chemotherapy in HER2-overexpressing patients who had not received prior chemotherapy for metastatic breast cancer. It is noteworthy that there was little difference (38% vs. 40%; $p=0.75$) in the objective response rate when Herceptin was added to chemotherapy in the 126 patients found to be FISH- as compared with a substantial difference (30% vs. 54%; $p<0.0001$) in the 325 patients noted to be FISH+.

Table 12

Overall Response Rate in Study H0648g for the Newly Defined Patient Subpopulations

	Herceptin +AC	AC Alone	Herceptin +Paclitaxel	Paclitaxel Alone	Herceptin +Chemo	Chemo Alone
FISH+						
n/N	57/99	37/87	32/65	12/74	89/164	49/161
%	58	42	49	16	54	30
95% CI	(47.8, 67.3)	(32.1, 52.9)	(37.1, 61.4)	(7.8, 24.6)	(46.6, 61.9)	(23.3, 37.5)
p-value (χ^2)	0.0406		<0.0001		<0.0001	
FISH-						
n/N	19/39	21/45	6/23	3/19	25/62	24/64
%	49	47	26	16	40	38
95% CI	(33.0, 64.4)	(32.1, 61.2)	(8.1, 44.0)	(0.0, 32.2)	(28.1, 52.5)	(25.6, 49.4)
p-value (χ^2)	0.8511		0.4182		0.7452	

Figures 4 and 5 show Kaplan-Meier curves for time to disease progression for the newly defined subpopulations. Although there is a marked improvement in time to disease progression for the FISH+ subpopulation (risk ratio of 0.44; p=0.0001), there is also a borderline improvement in time to disease progression for the FISH- subpopulation (risk ratio of 0.66; p=0.0429). (Note: The risk ratio represents the risk of progressing in the Herceptin + chemotherapy treatment group over that in the chemotherapy alone treatment group.)

Figure 4

Time to Disease Progression for FISH+ Patients in Study H0648g:
Herceptin + Chemotherapy vs. Chemotherapy Alone

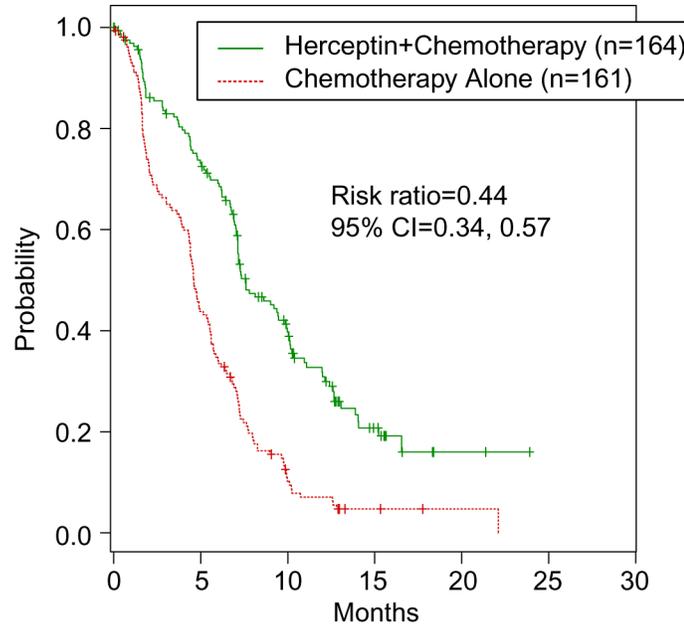
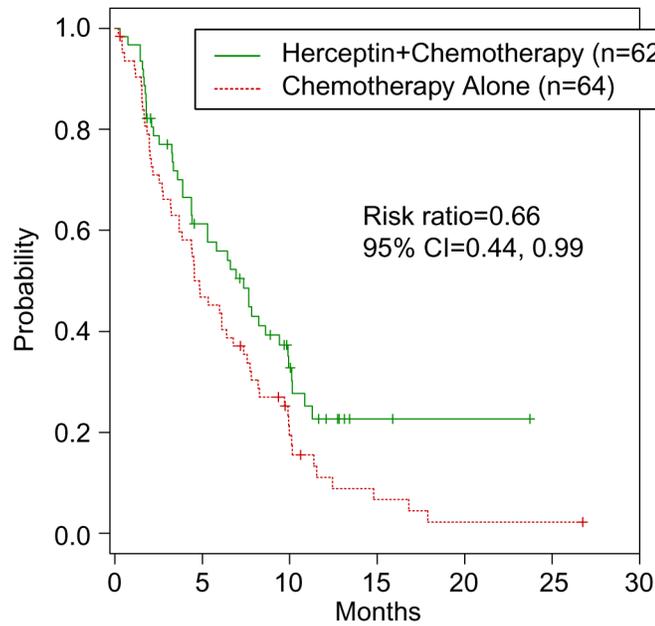


Figure 5

Time to Disease Progression for FISH- Patients in Study H0648g:
Herceptin + Chemotherapy vs. Chemotherapy Alone



Figures 6 and 7 show Kaplan-Meier curves for survival time for the two newly defined subpopulations. There is a marked improvement in survival for the FISH+ subpopulation (risk ratio of 0.69; $p = 0.007$) as compared with no survival benefit (risk ratio of 1.07; $p = 0.754$) in the FISH- subpopulation. These results were noted despite the crossover design, which permitted approximately 65% of control patients to receive Herceptin at the time of disease progression. (Note: The risk ratio represents the risk of death in the Herceptin + chemotherapy treatment group over that in the chemotherapy alone treatment group.)

Figure 6

Survival Time for FISH+ Patients in Study H0648g:
Herceptin + Chemotherapy vs. Chemotherapy Alone

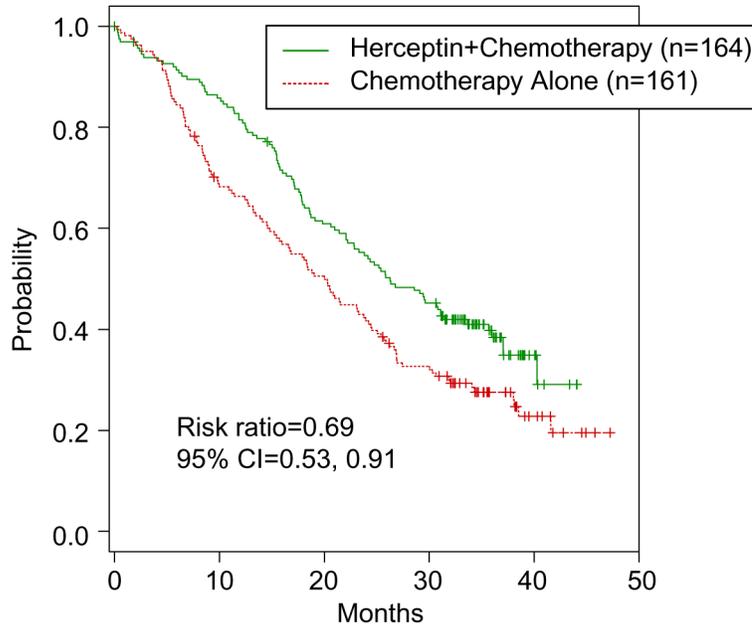
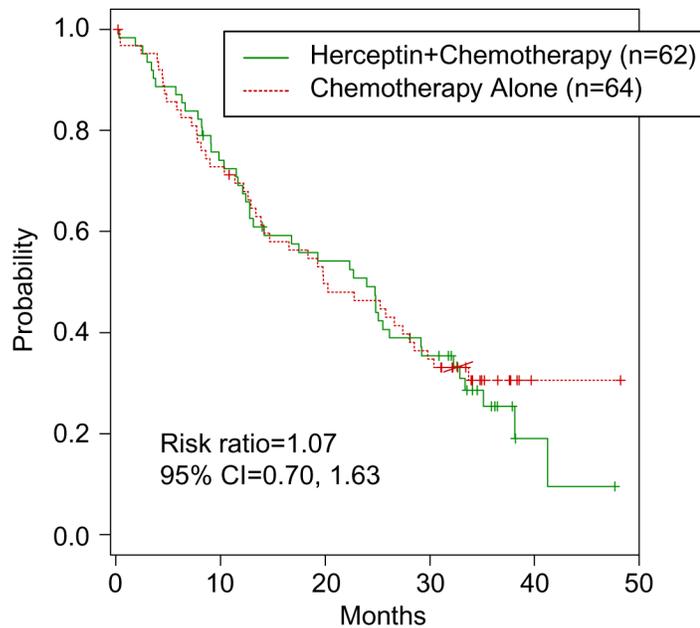


Figure 7

Survival Time for FISH- Patients in Study H0648g:
Herceptin + Chemotherapy vs. Chemotherapy Alone



4.8 INTERPRETATION AND CONCLUSIONS

Within each of these study populations (selected by overexpression of HER2 at the 2+ or 3+ level), selection by FISH identified a population that appears to benefit from Herceptin therapy. The single-agent trial, Study H0649g, demonstrated no responses among 46 patients lacking evidence of HER2 gene amplification, whereas the response rate in the FISH+ subpopulation was 20%. Within the randomized trial, Study H0648g, the same differences are apparent for the efficacy endpoints of objective response rate and survival. For the time to disease progression endpoint, although the clinical benefit appeared to be greater in the FISH+ than the FISH- subpopulation, there remained a benefit among FISH- patients.

In interpreting these results, the limitations of the analyses should be kept in mind. These were retrospective and exploratory observations with inadequate statistical power to make definitive conclusions. This was not a prospective clinical trial evaluating the benefit of Herceptin in a population of patients selected by FISH. Despite these limitations, these data, viewed in the context of the concordance analysis, support the conclusion that FISH is an appropriate alternative methodology to select patients for Herceptin therapy.

5. CONSIDERATIONS IN CONFIRMATORY PROSPECTIVE TRIALS

For the pivotal Herceptin clinical trials, HER2 overexpression was defined by an IHC assay specifically developed for the trials (the CTA). Since the conclusion of the pivotal Herceptin trials, new assays for testing HER2 protein overexpression/gene amplification have been developed. An important question involves the feasibility of obtaining reliable and conclusive clinical outcome data for patients selected for Herceptin therapy by the new assays.

The specific assay under consideration in this submission is PathVysion™. An overall concordance of 82% was demonstrated between the CTA and PathVysion™ (see Section 3.5) based on a sample with an equal proportion of CTA-positive and -negative tissues. Based on this concordance and the expected overall distribution of HER2

overexpression in women with metastatic breast cancer screened using the CTA, the extrapolated concordance is 88%. Of specimens testing FISH+ by PathVysion™, 91% would be expected to test at least 2+ in the CTA (see Section 3.6 for details).

The most conclusive and reliable data regarding the assessment of PathVysion™ as a method to select patients likely to benefit from Herceptin therapy would be generated through a prospective, randomized clinical trial. In the metastatic setting, given the survival benefit demonstrated for Herceptin in IHC 2+/3+ patients, ethical considerations would require that such a trial focus on the subgroup of patients who are FISH+ and IHC-. Based on the concordance analyses presented above, this subset of patients would constitute ~10% of all FISH+ patients (~2% of patients with metastatic breast cancer). Assuming a recruitment velocity similar to that in the pivotal trials, such a study (N=700 patients; endpoint of survival) would translate into an ~12-year accrual period.

Because HER2-positive patients (FISH+ or IHC+) would most likely choose not to participate in a randomized trial, Genentech also modeled a trial scenario in which all FISH+ or IHC+ patients would receive Herceptin + paclitaxel as first-line therapy for metastatic breast cancer. Two groups of patients (IHC-/FISH+ and IHC+/FISH-) would be evaluated. Since patients in both groups are HER2 overexpressors by either IHC or FISH, it is reasonable to expect that differences in clinical benefit would not be large. In order to demonstrate a >10% difference in clinical benefit (as measured by survival) between the two groups, a sample size of 4100 would be required, with a trial duration of >12 years.

In the adjuvant setting, patients could be ethically randomized to receive treatment with or without Herceptin since the risk/benefit ratio of treatment with Herceptin in this setting has not been established. There are currently three large trials of Herceptin in the adjuvant setting that are actively enrolling patients in the United States. In all of these trials, the primary endpoint is long-term disease-free survival (median survival time for the control arm is assumed to be 5 to 6 years). As the result of the relatively high concordance between IHC and FISH (>75%; see Section 3.5), the majority of patients enrolled in the adjuvant trials will be

HER2 positive by both assays (IHC and FISH). In spite of the large numbers of patients randomized in these trials (~10,000 patients randomized over 4 years), the number of patients with discordant results (IHC-/FISH+ or IHC+/FISH-) will be relatively small. Approximately 1000 patients in each discordant subgroup might be available if data from all three trials were combined (i.e., when a meta-analysis is performed). An efficacy analysis evaluating the effect of Herceptin in these discordant subgroups may be underpowered (~50% to 60% power depending on the disease-free survival in the control arm). However, there will be enough clinical outcome data to provide reliable estimates of clinical benefit in the two discordant subgroups. There will also be enough clinical outcome data to evaluate the benefit of Herceptin in the aggregate set of patients with HER2 gene amplification as measured by FISH (i.e., regardless of IHC status).

6. CONCLUSIONS

The fundamental biology of HER2 in breast cancer suggests that HER2 protein overexpression is a manifestation of HER2 gene amplification, and in an ideal environment, assays to detect either of these events would be fully concordant. Unfortunately, tissue fixation issues along with the complicated methodologies involved in both IHC and FISH assays have a negative impact on this ideal situation. Deterioration in concordance can occur even when these assays are performed in the most skilled, high-volume, centralized laboratories. This was demonstrated by the results of the original HercepTest[®]/CTA analysis, the HercepTest[®]/Pathway[™] analysis contained in the Pathway[™] PMA, and the PathVysion[™]/CTA analysis contained in this submission. Each of these efforts produced similar concordance in the 80%–90% range.

Published reports suggest that the lack of concordance can increase as these assays are exported to smaller, less-skilled, low-volume laboratories. Similar observations have been reported in the assessment of hormone receptor status to aid in the selection of patients for anti-estrogen therapy. Variability in pre-analytical procedures (fixation, etc.) and non-compliance with specific assay instructions (including use of

specified reagents, precise procedures, and inclusion of controls for assay validation) are common causes of assay error. Clinicians need to be alert to the issues associated with HER2 testing—regardless of methodology—and consider the results within the clinical context of the individual patient. Repeat testing using the same methodology or an alternative methodology may be required.

Bearing these issues in mind, the concordance data support the conclusion that use of PathVysion™ should provide clinicians similar information, as compared with the HercepTest® assay, with regard to potential clinical benefit from Herceptin therapy. Although the exploratory clinical outcome data were generated retrospectively and within a population of patients known to express HER2 at the 2+/3+ level by the CTA, they provide reassurance that FISH is able to select patients likely to benefit from Herceptin therapy.

In summary, the data presented indicate that detection of HER2 gene amplification by PathVysion™ is an appropriate method to aid in the selection of patients for Herceptin therapy. Genentech believes that the data contained in this submission support changes to the Herceptin product labeling that include information regarding PathVysion™ as an alternative method of patient selection.

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APPENDIX A
HERCEPTIN® Package Insert

Herceptin®

Trastuzumab

anti-HER2 monoclonal antibody

WARNINGS:**CARDIOMYOPATHY**

HERCEPTIN administration can result in the development of ventricular dysfunction and congestive heart failure. Left ventricular function should be evaluated in all patients prior to and during treatment with HERCEPTIN. Discontinuation of HERCEPTIN treatment should be strongly considered in patients who develop a clinically significant decrease in left ventricular function. The incidence and severity of cardiac dysfunction was particularly high in patients who received HERCEPTIN in combination with anthracyclines and cyclophosphamide. (See WARNINGS.)

HYPERSENSITIVITY REACTIONS INCLUDING ANAPHYLAXIS**INFUSION REACTIONS****PULMONARY EVENTS**

HERCEPTIN administration can result in severe hypersensitivity reactions (including anaphylaxis), infusion reactions, and pulmonary events. Rarely, these have been fatal. In most cases, symptoms occurred during or within 24 hours of administration of HERCEPTIN. HERCEPTIN infusion should be interrupted for patients experiencing dyspnea or clinically significant hypotension. Patients should be monitored until signs and symptoms completely resolve. Discontinuation of HERCEPTIN treatment should be strongly considered for patients who develop anaphylaxis, angioedema, or acute respiratory distress syndrome. (See WARNINGS.)

DESCRIPTION

HERCEPTIN (Trastuzumab) is a recombinant DNA-derived humanized monoclonal antibody that selectively binds with high affinity in a cell-based assay ($K_d = 5$ nM) to the extracellular domain of the human epidermal growth factor receptor 2 protein, HER2.^{1,2} The antibody is an IgG₁ kappa that contains human framework regions with the complementarity-determining regions of a murine antibody (4D5) that binds to HER2.

The humanized antibody against HER2 is produced by a mammalian cell (Chinese Hamster Ovary) [CHO] suspension culture in a nutrient medium containing the antibiotic gentamicin. Gentamicin is not detectable in the final product.

HERCEPTIN is a sterile, white to pale yellow, preservative-free lyophilized powder for intravenous (IV) administration. The nominal content of each HERCEPTIN vial is 440 mg Trastuzumab, 9.9 mg L-histidine HCl, 6.4 mg L-histidine, 400 mg α, α -trehalose dihydrate, and 1.8 mg polysorbate 20. USP. Reconstitution with **only 20 mL of the supplied Bacteriostatic Water for Injection (BWF1)**, USP, containing 1.1% benzyl alcohol as a preservative, yields a multi-dose solution containing 21 mg/mL Trastuzumab, at a pH of approximately 6.

CLINICAL PHARMACOLOGY**General**

The HER2 (or c-erbB2) proto-oncogene encodes a transmembrane receptor protein of 185 kDa, which is structurally related to the epidermal growth factor receptor.¹ HER2 protein overexpression is observed in 25%–30% of primary breast cancers. HER2 protein overexpression can be determined using an immunohistochemistry-based assessment of fixed tumor blocks.³

Trastuzumab has been shown, in both *in vitro* assays and in animals, to inhibit the proliferation of human tumor cells that overexpress HER2.^{4,6}

Trastuzumab is a mediator of antibody-dependent cellular cytotoxicity (ADCC).^{7,8} *In vitro*, HERCEPTIN-mediated ADCC has been shown to be preferentially exerted on HER2 overexpressing cancer cells compared with cancer cells that do not overexpress HER2.

Pharmacokinetics

The pharmacokinetics of Trastuzumab were studied in breast cancer patients with metastatic disease. Short duration intravenous infusions of 10 to 500 mg once weekly demonstrated dose-dependent pharmacokinetics. Mean half-life increased and clearance decreased with increasing dose level. The half-life averaged 1.7 and 12 days at the 10 and 500 mg dose levels, respectively. Trastuzumab's volume of distribution was approximately that of serum volume (44 mL/kg). At the highest weekly dose studied (500 mg), mean peak serum concentrations were 377 microgram/mL.

In studies using a loading dose of 4 mg/kg followed by a weekly maintenance dose of 2 mg/kg, a mean half-life of 5.8 days (range = 1 to 32 days) was observed. Between Weeks 16 and 32, Trastuzumab serum concentrations reached a steady state with mean trough and peak concentrations of approximately 79 microgram/mL and 123 microgram/mL, respectively.

Detectable concentrations of the circulating extracellular domain of the HER2 receptor (shed antigen) are found in the serum of some patients with HER2 overexpressing tumors. Determination of shed antigen in baseline serum samples revealed that 64% (286/447) of patients had detectable shed antigen, which ranged as high as 1880 ng/mL (median = 11 ng/mL). Patients with higher baseline shed antigen levels were more likely to have lower serum trough concentrations. However, with weekly dosing, most patients with elevated shed antigen levels achieved target serum concentrations of Trastuzumab by Week 6.

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Data suggest that the disposition of Trastuzumab is not altered based on age or serum creatinine (up to 2.0 mg/dL). No formal interaction studies have been performed.

Mean serum trough concentrations of Trastuzumab, when administered in combination with paclitaxel, were consistently elevated approximately 1.5-fold as compared with serum concentrations of Trastuzumab used in combination with anthracycline plus cyclophosphamide. In primate studies, administration of Trastuzumab with paclitaxel resulted in a reduction in Trastuzumab clearance. Serum levels of Trastuzumab in combination with cisplatin, doxorubicin or epirubicin plus cyclophosphamide did not suggest any interactions; no formal drug interaction studies were performed.

CLINICAL STUDIES

The safety and efficacy of HERCEPTIN were studied in a randomized, controlled clinical trial in combination with chemotherapy (469 patients) and an open-label single agent clinical trial (222 patients). Both trials studied patients with metastatic breast cancer whose tumors overexpress the HER2 protein. Patients were eligible if they had 2+ or 3+ levels of overexpression (based on a 0–3+ scale) by immunohistochemical assessment of tumor tissue performed by a central testing lab.

A multicenter, randomized, controlled clinical trial was conducted in 469 patients with metastatic breast cancer who had not been previously treated with chemotherapy for metastatic disease. Patients were randomized to receive chemotherapy alone or in combination with HERCEPTIN given intravenously as a 4 mg/kg loading dose followed by weekly doses of HERCEPTIN at 2 mg/kg. For those who had received prior anthracycline therapy in the adjuvant setting, chemotherapy consisted of paclitaxel (175 mg/m² over 3 hours every 21 days for at least six cycles); for all other patients, chemotherapy consisted of anthracycline plus cyclophosphamide (AC: doxorubicin 60 mg/m² or epirubicin 75 mg/m² plus 600 mg/m² cyclophosphamide every 21 days for six cycles). Compared with patients in the AC subgroups (n = 281), patients in the paclitaxel subgroups (n = 188) were more likely to have had the following: poor prognostic factors (premenopausal status, estrogen or progesterone receptor negative tumors, positive lymph nodes), prior therapy (adjuvant chemotherapy, myeloablative chemotherapy, radiotherapy), and a shorter disease-free interval.

Compared with patients randomized to chemotherapy alone, the patients randomized to HERCEPTIN and chemotherapy experienced a significantly longer median time to disease progression, a higher overall response rate (ORR), a longer median duration of response, and a higher one-year survival rate. (See Table 1.) These treatment effects were observed both in patients who received HERCEPTIN plus paclitaxel and in those who received HERCEPTIN plus AC, however the magnitude of the effects was greater in the paclitaxel subgroup. The degree of HER2 overexpression was a predictor of treatment effect. (See CLINICAL STUDIES: *HER2 protein overexpression*.)

Table 1
Phase III Clinical Efficacy in First-Line Treatment

	Combined Results HERCEPTIN		Paclitaxel subgroup HERCEPTIN		AC subgroup HERCEPTIN	
	All Chemo- therapy (n = 235)	All Chemo- therapy (n = 234)	Paclitaxel (n = 92)	Paclitaxel (n = 96)	AC ^a (n = 143)	AC (n = 138)
Primary Endpoint						
Time to Progression ^{b,c}						
Median (months)	7.2	4.5	6.7	2.5	7.6	5.7
95% confidence interval	6.9, 8.2	4.3, 4.9	5.2, 9.9	2.0, 4.3	7.2, 9.1	4.6, 7.1
p-value (log rank)		<0.0001		<0.0001		0.002
Secondary Endpoints						
Overall Response Rate ^b						
Rate (percent)	45	29	38	15	50	38
95% confidence interval	39, 51	23, 35	28, 48	8, 22	42, 58	30, 46
p-value (Z-test)		<0.001		<0.001		0.10
Duration of Response ^{b,c}						
Median (months)	8.3	5.8	8.3	4.3	8.4	6.4
25%, 75% quantile	5.5, 14.8	3.9, 8.5	5.1, 11.0	3.7, 7.4	5.8, 14.8	4.5, 8.5
1-Year Survival ^c						
Percent alive	79	68	73	61	83	73
95% confidence interval	74, 84	62, 74	66, 80	51, 71	77, 89	66, 82
p-value (Z-test)		<0.01		0.08		0.04

^a AC = anthracycline (doxorubicin or epirubicin) and cyclophosphamide.

^b Assessed by an independent Response Evaluation Committee.

^c Kaplan-Meier Estimate

HERCEPTIN was studied as a single agent in a multicenter, open-label, single-arm clinical trial in patients with HER2 overexpressing metastatic breast cancer who had relapsed following one or two prior chemotherapy regimens for metastatic disease. Of 222 patients enrolled, 66% had received prior adjuvant chemotherapy, 68% had received two prior chemotherapy regimens for metastatic disease, and 25% had received prior myeloablative treatment with hematopoietic rescue. Patients were treated with a loading dose of 4 mg/kg IV followed by weekly doses of HERCEPTIN at 2 mg/kg IV. The ORR (complete response + partial response), as determined by an independent Response Evaluation Committee, was 14%, with a 2% complete response rate and a 12% partial response rate. Complete responses were observed only in patients with disease limited to skin and lymph nodes. The degree of HER2 overexpression was a predictor of treatment effect. (See CLINICAL STUDIES: *HER2 protein overexpression*.)

HER2 protein overexpression

Relationship to Response: In the clinical studies described, patient eligibility was determined by testing tumor specimens for overexpression of HER2 protein. Specimens were

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tested with a research-use-only immunohistochemical assay (referred to as the Clinical Trial Assay, CTA) and scored as 0, 1+, 2+, or 3+ with 3+ indicating the strongest positivity. Only patients with 2+ or 3+ positive tumors were eligible (about 33% of those screened).

Data from both efficacy trials suggest that the beneficial treatment effects were largely limited to patients with the highest level of HER2 protein overexpression (3+). (See Table 2.)

Table 2
Treatment Effect versus Level of HER2 Expression

	Single-Arm Trial HERCEPTIN	Treatment Subgroups in Randomized Trial			
		HERCEPTIN + Paclitaxel	Paclitaxel	HERCEPTIN + AC	AC
Overall Response Rate					
2+ overexpression	4% (2/50)	21% (5/24)	16% (3/19)	40% (14/35)	43% (18/42)
3+ overexpression	17% (29/172)	44% (30/68)	14% (11/77)	53% (57/108)	36% (35/96)
Median time to progression (months) (95% CI)					
2+ overexpression	N/A ^a	4.4 (2.2, 6.6)	3.2 (2.0, 5.6)	7.8 (6.4, 10.1)	7.1 (4.8, 9.8)
3+ overexpression	N/A ^a	7.1 (6.2, 12.0)	2.2 (1.8, 4.3)	7.3 (7.1, 9.2)	4.9 (4.5, 6.9)

^aN/A = Not Assessed

Immunohistochemical Detection: In clinical trials, the Clinical Trial Assay (CTA) was used for immunohistochemical detection of HER2 protein overexpression. The DAKO HercepTest™, another immunohistochemical test for HER2 protein overexpression, has not been directly studied for its ability to predict HERCEPTIN treatment effect, but has been compared to the CTA on over 500 breast cancer histology specimens obtained from the National Cancer Institute Cooperative Breast Cancer Tissue Resource. Based upon these results and an expected incidence of 33% of 2+ or 3+ HER2 overexpression in tumors from women with metastatic breast cancer, one can estimate the correlation of the HercepTest™ results with CTA results. Of specimens testing 3+ (strongly positive) on the HercepTest™, 94% would be expected to test at least 2+ on the CTA (i.e., meeting the study entry criterion) including 82% which would be expected to test 3+ on the CTA (i.e., the reading most associated with clinical benefit). Of specimens testing 2+ (weakly positive) on the HercepTest™, only 34% would be expected to test at least 2+ on the CTA, including 14% which would be expected to test 3+ on the CTA.

INDICATIONS AND USAGE

HERCEPTIN as a single agent is indicated for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein and who have received one or more chemotherapy regimens for their metastatic disease. HERCEPTIN in combination with paclitaxel is indicated for treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein and who have not received chemotherapy for their metastatic disease. HERCEPTIN should only be used in patients whose tumors have HER2 protein overexpression. (See CLINICAL STUDIES: *HER2 protein overexpression* for information regarding HER2 protein testing and the relationship between the degree of overexpression and the treatment effect.)

CONTRAINDICATIONS

None known.

WARNINGS

Cardiotoxicity:

Signs and symptoms of cardiac dysfunction, such as dyspnea, increased cough, paroxysmal nocturnal dyspnea, peripheral edema, S₃ gallop, or reduced ejection fraction, have been observed in patients treated with HERCEPTIN. Congestive heart failure associated with HERCEPTIN therapy may be severe and has been associated with disabling cardiac failure, death, and mural thrombosis leading to stroke. The clinical status of patients in the trials who developed congestive heart failure was classified for severity using the New York Heart Association classification system (I–IV, where IV is the most severe level of cardiac failure). (See Table 3.)

Table 3
Incidence and Severity of Cardiac Dysfunction

	HERCEPTIN ^a alone n = 213	HERCEPTIN+ Paclitaxel ^b n = 91	Paclitaxel ^b n = 95	HERCEPTIN+ Anthracycline+ cyclophosphamide ^b n = 143	Anthracycline+ cyclophosphamide ^b n = 135
Any Cardiac Dysfunction	7%	11%	1%	28%	7%
Class III-IV	5%	4%	1%	19%	3%

^a Open-label, single-agent Phase II study (94% received prior anthracyclines).

^b Randomized Phase III study comparing chemotherapy plus HERCEPTIN to chemotherapy alone, where chemotherapy is either anthracycline/cyclophosphamide or paclitaxel.

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Candidates for treatment with HERCEPTIN should undergo thorough baseline cardiac assessment including history and physical exam and one or more of the following: EKG, echocardiogram, and MUGA scan. There are no data regarding the most appropriate method of evaluation for the identification of patients at risk for developing cardiotoxicity. Monitoring may not identify all patients who will develop cardiac dysfunction.

Extreme caution should be exercised in treating patients with pre-existing cardiac dysfunction.

Patients receiving HERCEPTIN should undergo frequent monitoring for deteriorating cardiac function.

The probability of cardiac dysfunction was highest in patients who received HERCEPTIN concurrently with anthracyclines. The data suggest that advanced age may increase the probability of cardiac dysfunction.

Pre-existing cardiac disease or prior cardiotoxic therapy (e.g., anthracycline or radiation therapy to the chest) may decrease the ability to tolerate HERCEPTIN therapy; however, the data are not adequate to evaluate the correlation between HERCEPTIN-induced cardiotoxicity and these factors.

Discontinuation of HERCEPTIN therapy should be strongly considered in patients who develop clinically significant congestive heart failure. In the clinical trials, most patients with cardiac dysfunction responded to appropriate medical therapy often including discontinuation of HERCEPTIN. The safety of continuation or resumption of HERCEPTIN in patients who have previously experienced cardiac toxicity has not been studied. There are insufficient data regarding discontinuation of HERCEPTIN therapy in patients with asymptomatic decreases in ejection fraction; such patients should be closely monitored for evidence of clinical deterioration.

Hypersensitivity Reactions Including Anaphylaxis:

Severe hypersensitivity reactions have been infrequently reported in patients treated with HERCEPTIN. Signs and symptoms include anaphylaxis, urticaria, bronchospasm, angioedema, and/or hypotension. In some cases, the reactions have been fatal. The onset of symptoms generally occurred during an infusion, but there have also been reports of symptom onset after the completion of an infusion. Reactions were most commonly reported in association with the initial infusion.

HERCEPTIN infusion should be interrupted in all patients with severe hypersensitivity reactions. In the event of a hypersensitivity reaction, appropriate medical therapy should be administered, which may include epinephrine, corticosteroids, diphenhydramine, bronchodilators, and oxygen. Patients should be evaluated and carefully monitored until complete resolution of signs and symptoms.

There are no data regarding the most appropriate method of identification of patients who may safely be retreated with HERCEPTIN after experiencing a severe hypersensitivity reaction. HERCEPTIN has been readministered to some patients who fully recovered from a previous severe reaction. Prior to readministration of HERCEPTIN, the majority of these patients were prophylactically treated with pre-medications including antihistamines and/or corticosteroids. While some of these patients tolerated retreatment, others had severe reactions again despite the use of prophylactic pre-medications.

Infusion Reactions:

In the postmarketing setting, rare occurrences of severe infusion reactions leading to a fatal outcome have been associated with the use of HERCEPTIN.

In clinical trials, infusion reactions consisted of a symptom complex characterized by fever and chills, and on occasion included nausea, vomiting, pain (in some cases at tumor sites), headache, dizziness, dyspnea, hypotension, rash, and asthenia. These reactions were usually mild to moderate in severity. (See ADVERSE REACTIONS.)

However, in postmarketing reports, more severe adverse reactions to HERCEPTIN infusion were observed and included bronchospasm, hypoxia, and severe hypotension. These severe reactions were usually associated with the initial infusion of HERCEPTIN and generally occurred during or immediately following the infusion. However, the onset and clinical course were variable. For some patients, symptoms progressively worsened and led to further pulmonary complications. (See PULMONARY EVENTS section of WARNINGS.) In other patients with acute onset of signs and symptoms, initial improvement was followed by clinical deterioration. Delayed post-infusion events with rapid clinical deterioration have also been reported. Rarely, severe infusion reactions culminated in death within hours or up to one week following an infusion.

Some severe reactions have been treated successfully with interruption of the HERCEPTIN infusion and administration of supportive therapy including oxygen, intravenous fluids, beta-agonists, and corticosteroids.

There are no data regarding the most appropriate method of identification of patients who may safely be retreated with HERCEPTIN after experiencing a severe infusion reaction. HERCEPTIN has been readministered to some patients who fully recovered from the previous severe reaction. Prior to readministration of HERCEPTIN, the majority of these patients were prophylactically treated with pre-medications including antihistamines and/or corticosteroids. While some of these patients tolerated retreatment, others had severe reactions again despite the use of prophylactic pre-medications.

Pulmonary Events:

Severe pulmonary events leading to death have been reported rarely with the use of HERCEPTIN in the postmarketing setting. Signs, symptoms, and clinical findings include dyspnea, pulmonary infiltrates, pleural effusions, non-cardiogenic pulmonary edema, pulmonary insufficiency and hypoxia, and acute respiratory distress syndrome. These events may or may not occur as sequelae of infusion reactions. (See INFUSION REACTIONS section of WARNINGS.) Patients with symptomatic intrinsic lung disease or with extensive tumor

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involvement of the lungs, resulting in dyspnea at rest, may be at greater risk of severe reactions. Other severe events reported rarely in the postmarketing setting include pneumonitis and pulmonary fibrosis.

PRECAUTIONS

General: HERCEPTIN therapy should be used with caution in patients with known hypersensitivity to Trastuzumab, Chinese Hamster Ovary cell proteins, or any component of this product.

Patients with Cardiac Ventricular Dysfunction

Extreme caution should be exercised in treating patients with pre-existing cardiac dysfunction. (See WARNINGS.)

Patients with Pulmonary Disorders

Patients with either symptomatic intrinsic pulmonary disease (e.g., asthma, COPD) or patients with extensive tumor involvement of the lungs (e.g., lymphangitic spread of tumor, pleural effusions, parenchymal masses), resulting in dyspnea at rest, may be at increased risk for severe pulmonary adverse events. (See WARNINGS.)

Drug Interactions: There have been no formal drug interaction studies performed with HERCEPTIN in humans. Administration of paclitaxel in combination with HERCEPTIN resulted in a two-fold decrease in HERCEPTIN clearance in a non-human primate study and in a 1.5-fold increase in HERCEPTIN serum levels in clinical studies. (See PHARMACOKINETICS.)

Benzyl Alcohol: For patients with a known hypersensitivity to benzyl alcohol (the preservative in Bacteriostatic Water for Injection) reconstitute HERCEPTIN with Sterile Water for Injection (SWFI). USP. DISCARD THE SWFI-RECONSTITUTED HERCEPTIN VIAL FOLLOWING A SINGLE USE.

Immunogenicity: Of 903 patients who have been evaluated, human anti-human antibody (HAHA) to Trastuzumab was detected in one patient, who had no allergic manifestations.

Carcinogenesis, Mutagenesis, Impairment of Fertility:

Carcinogenesis: HERCEPTIN has not been tested for its carcinogenic potential.

Mutagenesis: No evidence of mutagenic activity was observed in Ames tests using six different test strains of bacteria, with and without metabolic activation, at concentrations of up to 5000 µg/mL Trastuzumab. Human peripheral blood lymphocytes treated *in vitro* at concentrations of up to 5000 µg/plate Trastuzumab, with and without metabolic activation, revealed no evidence of mutagenic potential. In an *in vivo* mutagenic assay (the micronucleus assay), no evidence of chromosomal damage to mouse bone marrow cells was observed following bolus intravenous doses of up to 118 mg/kg Trastuzumab.

Impairment of Fertility: A fertility study has been conducted in female cynomolgus monkeys at doses up to 25 times the weekly human maintenance dose of 2 mg/kg HERCEPTIN and has revealed no evidence of impaired fertility.

Pregnancy Category B: Reproduction studies have been conducted in cynomolgus monkeys at doses up to 25 times the weekly human maintenance dose of 2 mg/kg HERCEPTIN and have revealed no evidence of impaired fertility or harm to the fetus. However, HER2 protein expression is high in many embryonic tissues including cardiac and neural tissues; in mutant mice lacking HER2, embryos died in early gestation.* Placental transfer of HERCEPTIN during the early (Days 20–50 of gestation) and late (Days 120–150 of gestation) fetal development period was observed in monkeys. There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, this drug should be used during pregnancy only if clearly needed.

Nursing Mothers: A study conducted in lactating cynomolgus monkeys at doses 25 times the weekly human maintenance dose of 2 mg/kg HERCEPTIN demonstrated that Trastuzumab is secreted in the milk. The presence of Trastuzumab in the serum of infant monkeys was not associated with any adverse effects on their growth or development from birth to 3 months of age. It is not known whether HERCEPTIN is excreted in human milk. Because human IgG is excreted in human milk, and the potential for absorption and harm to the infant is unknown, women should be advised to discontinue nursing during HERCEPTIN therapy and for 6 months after the last dose of HERCEPTIN.

Pediatric Use: The safety and effectiveness of HERCEPTIN in pediatric patients have not been established.

Geriatric Use: HERCEPTIN has been administered to 133 patients who were 65 years of age or over. The risk of cardiac dysfunction may be increased in geriatric patients. The reported clinical experience is not adequate to determine whether older patients respond differently from younger patients.

ADVERSE REACTIONS

In clinical studies, a total of 958 patients have received HERCEPTIN alone or in combination with chemotherapy. Data in Table 4 are based on the experience with the recommended dosing regimen for HERCEPTIN in the randomized controlled clinical trial in 234 patients who received HERCEPTIN in combination with chemotherapy and four open-label studies of HERCEPTIN as a single agent in 352 patients at doses of 10–500 mg administered weekly.

Cardiac Failure/Dysfunction: For a description of cardiac toxicities, see WARNINGS.

Anemia and Leukopenia: An increased incidence of anemia and leukopenia was observed in the treatment group receiving HERCEPTIN and chemotherapy, especially in the HERCEPTIN and AC subgroup, compared with the treatment group receiving chemotherapy alone. The majority of these cytopenic events were mild or moderate in intensity, reversible, and none resulted in discontinuation of therapy with HERCEPTIN.

Hematologic toxicity is infrequent following the administration of HERCEPTIN as a single

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agent, with an incidence of Grade III toxicities for WBC, platelets, hemoglobin all < 1%. No Grade IV toxicities were observed.

Diarrhea: Of patients treated with HERCEPTIN as a single agent, 25% experienced diarrhea. An increased incidence of diarrhea, primarily mild to moderate in severity, was observed in patients receiving HERCEPTIN in combination with chemotherapy.

Infection: An increased incidence of infections, primarily mild upper respiratory infections of minor clinical significance or catheter infections, was observed in patients receiving HERCEPTIN in combination with chemotherapy.

Infusion Reactions: During the first infusion with HERCEPTIN, a symptom complex most commonly consisting of chills and/or fever was observed in about 40% of patients in clinical trials. The symptoms were usually mild to moderate in severity and were treated with acetaminophen, diphenhydramine, and meperidine (with or without reduction in the rate of HERCEPTIN infusion). HERCEPTIN discontinuation was infrequent. Other signs and/or symptoms may include nausea, vomiting, pain (in some cases at tumor sites), rigors, headache, dizziness, dyspnea, hypotension, rash, and asthenia. The symptoms occurred infrequently with subsequent HERCEPTIN infusions. (See WARNINGS for information on more severe reactions reported in the postmarketing setting.)

Hypersensitivity Reactions Including Anaphylaxis

Pulmonary Events:

In the postmarketing setting, severe hypersensitivity reactions (including anaphylaxis), infusion reactions, and pulmonary adverse events have been reported. These events include anaphylaxis, angioedema, bronchospasm, hypotension, hypoxia, dyspnea, pulmonary infiltrates, pleural effusions, non-cardiogenic pulmonary edema, and acute respiratory distress syndrome. For a detailed description, see WARNINGS.

Table 4
Adverse Events Occurring in ≥ 5% of Patients or at Increased Incidence in the HERCEPTIN Arm of the Randomized Study (Percent of Patients)

	Single Agent n = 352	HERCEPTIN + Paclitaxel n = 91	Paclitaxel Alone n = 95	HERCEPTIN + AC n = 143	AC Alone n = 135
Body as a Whole					
Pain	47	61	62	57	42
Asthenia	42	62	57	54	55
Fever	36	49	23	56	34
Chills	32	41	4	35	11
Headache	26	36	28	44	31
Abdominal pain	22	34	22	23	18
Back pain	22	34	30	27	15
Infection	20	47	27	47	31
Flu syndrome	10	12	5	12	6
Accidental injury	6	13	3	9	4
Allergic reaction	3	8	2	4	2
Cardiovascular					
Tachycardia	5	12	4	10	5
Congestive heart failure	7	11	1	28	7
Digestive					
Nausea	33	51	9	76	77
Diarrhea	25	45	29	45	26
Vomiting	23	37	28	53	49
Nausea and vomiting	8	14	11	18	9
Anorexia	14	24	16	31	26
Heme & Lymphatic					
Anemia	4	14	9	36	26
Leukopenia	3	24	17	52	34
Metabolic					
Peripheral edema	10	22	20	20	17
Edema	8	10	8	11	5
Musculoskeletal					
Bone pain	7	24	18	7	7
Arthralgia	6	37	21	8	9
Nervous					
Insomnia	14	25	13	29	15
Dizziness	13	22	24	24	18
Paresthesia	9	48	39	17	11
Depression	6	12	13	20	12
Peripheral neuritis	2	23	16	2	2
Neuropathy	1	13	5	4	4
Respiratory					
Cough increased	26	41	22	43	29
Dyspnea	22	27	26	42	25
Rhinitis	14	22	5	22	16
Pharyngitis	12	22	14	30	18
Sinusitis	9	21	7	13	6
Skin					
Rash	18	38	18	27	17
Herpes simplex	2	12	3	7	9
Acne	2	11	3	3	< 1
Urogenital					
Urinary tract infection	5	18	14	13	7

HERCEPTIN® (Trastuzumab)**Other serious adverse events**

The following other serious adverse events occurred in at least one of the 958 patients treated with HERCEPTIN in clinical studies:

Body as a Whole: cellulitis, anaphylactoid reaction, ascites, hydrocephalus, radiation injury, deafness, amblyopia

Cardiovascular: vascular thrombosis, pericardial effusion, heart arrest, hypotension, syncope, hemorrhage, shock, arrhythmia

Digestive: hepatic failure, gastroenteritis, hematemesis, ileus, intestinal obstruction, colitis, esophageal ulcer, stomatitis, pancreatitis, hepatitis

Endocrine: hypothyroidism

Hematological: pancytopenia, acute leukemia, coagulation disorder, lymphangitis

Metabolic: hypercalcemia, hypomagnesemia, hyponatremia, hypoglycemia, growth retardation, weight loss

Musculoskeletal: pathological fractures, bone necrosis, myopathy

Nervous: convulsion, ataxia, confusion, manic reaction

Respiratory: apnea, pneumothorax, asthma, hypoxia, laryngitis

Skin: herpes zoster, skin ulceration

Urogenital: hydronephrosis, kidney failure, cervical cancer, hematuria, hemorrhagic cystitis, pyelonephritis

OVERDOSAGE

There is no experience with overdosage in human clinical trials. Single doses higher than 500 mg have not been tested.

DOSAGE AND ADMINISTRATION**Usual Dose**

The recommended initial loading dose is 4 mg/kg Trastuzumab administered as a 90-minute infusion. The recommended weekly maintenance dose is 2 mg/kg Trastuzumab and can be administered as a 30-minute infusion if the initial loading dose was well tolerated. HERCEPTIN may be administered in an outpatient setting. HERCEPTIN is to be diluted in saline for IV infusion. **DO NOT ADMINISTER AS AN IV PUSH OR BOLUS.** (See ADMINISTRATION.)

Preparation for Administration

The diluent provided has been formulated to maintain the stability and sterility of HERCEPTIN for up to 28 days. Other diluents have not been shown to contain effective preservatives for HERCEPTIN. Each vial of HERCEPTIN should be reconstituted with **ONLY 20 mL of BWFI, USP, 1.1% benzyl alcohol preserved, as supplied**, to yield a multi-dose solution containing 21 mg/mL Trastuzumab. Use of all 30 mL of diluent results in a lower-than-intended dose of HERCEPTIN. THE REMAINDER (approximately 10 mL) OF THE DILUENT SHOULD BE DISCARDED. Immediately upon reconstitution with BWFI, the vial of HERCEPTIN must be labeled in the area marked "Do not use after:" with the future date that is 28 days from the date of reconstitution.

If the patient has known hypersensitivity to benzyl alcohol, HERCEPTIN must be reconstituted with Sterile Water for Injection. (See PRECAUTIONS.) HERCEPTIN WHICH HAS BEEN RECONSTITUTED WITH SWFI MUST BE USED IMMEDIATELY AND ANY UNUSED PORTION DISCARDED. USE OF OTHER RECONSTITUTION DILUENTS SHOULD BE AVOIDED.

Shaking the reconstituted HERCEPTIN or causing excessive foaming during the addition of diluent may result in problems with dissolution and the amount of HERCEPTIN that can be withdrawn from the vial.

Use appropriate aseptic technique when performing the following reconstitution steps:

- Using a sterile syringe, slowly inject **20 mL** of the diluent into the vial containing the lyophilized cake of Trastuzumab. The stream of diluent should be directed into the lyophilized cake.
- Swirl the vial gently to aid reconstitution. Trastuzumab may be sensitive to shear-induced stress, e.g., agitation or rapid expulsion from a syringe. **DO NOT SHAKE.**
- Slight foaming of the product upon reconstitution is not unusual. Allow the vial to stand undisturbed for approximately 5 minutes. The solution should be essentially free of visible particulates, clear to slightly opalescent, and colorless to pale yellow.

Determine the number of mg of Trastuzumab needed, based on a loading dose of 4 mg Trastuzumab/kg body weight or a maintenance dose of 2 mg Trastuzumab/kg body weight. Calculate the volume of 21 mg/mL Trastuzumab solution and withdraw this amount from the vial and add it to an infusion bag containing 250 mL of 0.9% Sodium Chloride Injection, USP. **DEXTROSE (5%) SOLUTION SHOULD NOT BE USED.** Gently invert the bag to mix the solution. The reconstituted preparation results in a colorless to pale yellow transparent solution. Parenteral drug products should be inspected visually for particulates and discoloration prior to administration.

No incompatibilities between HERCEPTIN and polyvinylchloride or polyethylene bags have been observed.

Administration

Treatment may be administered in an outpatient setting by administration of a 4 mg/kg Trastuzumab loading dose by intravenous (IV) infusion over 90 minutes. **DO NOT ADMINISTER AS AN IV PUSH OR BOLUS.** Patients should be observed for fever and chills or other infusion-associated symptoms. (See ADVERSE REACTIONS.) If prior infusions are well tolerated, subsequent weekly doses of 2 mg/kg Trastuzumab may be administered over 30 minutes.

HERCEPTIN® (Trastuzumab)

HERCEPTIN should not be mixed or diluted with other drugs. HERCEPTIN infusions should not be administered or mixed with Dextrose solutions.

Stability and Storage

Vials of HERCEPTIN are stable at 2–8°C (36–46°F) prior to reconstitution. Do not use beyond the expiration date stamped on the vial. A vial of HERCEPTIN reconstituted with BWFI, as supplied, is stable for 28 days after reconstitution when stored refrigerated at 2–8°C (36–46°F), and the solution is preserved for multiple use. Discard any remaining multi-dose reconstituted solution after 28 days. If unpreserved SWFI (not supplied) is used, the reconstituted HERCEPTIN solution should be used immediately and any unused portion must be discarded. **DO NOT FREEZE HERCEPTIN THAT HAS BEEN RECONSTITUTED.**

The solution of HERCEPTIN for infusion diluted in polyvinylchloride or polyethylene bags containing 0.9% Sodium Chloride Injection, USP, may be stored at 2–8°C (36–46°F) for up to 24 hours prior to use. Diluted HERCEPTIN has been shown to be stable for up to 24 hours at room temperature (2–25°C). However, since diluted HERCEPTIN contains no effective preservative, the reconstituted and diluted solution should be stored refrigerated (2–8°C).

HOW SUPPLIED

HERCEPTIN is supplied as a lyophilized, sterile powder nominally containing 440 mg Trastuzumab per vial under vacuum.

Each carton contains one vial of 440 mg HERCEPTIN (Trastuzumab) and one 30 mL vial of Bacteriostatic Water for Injection, USP, 1.1% benzyl alcohol. NDC 50242-134-60.

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HERCEPTIN® (Trastuzumab)

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APPENDIX B
Additional Concordance Analyses

The results of the concordance analyses for HercepTest[®] and PathVysion[™] that provided the basis for FDA approval are presented in Tables 1 and 3.

Table 1
HercepTest[®]/CTA Concordance

HercepTest [®]	CTA Score				Total
	0	1+	2+	3+	
0	96	20	20	3	139
1+	72	27	30	5	134
2+	24	29	57	16	126
3+	3	3	36	107	149
Total	195	79	143	131	548
Percentage	36	14	26	24	100

Note: Concordance, 79% (95% CI of 76%, 82%). κ statistic: 0.72 (95% CI of 0.58, 0.7).

Source: HercepTest[®] PMA.

Table 2
Extrapolated HercepTest[®]/CTA Concordance

HercepTest [®]	CTA Score				Total
	0	1+	2+	3+	
0	157	12	8	3	180
1+	117	16	12	5	150
2+	39	18	22	15	94
3+	5	2	14	103	124
Total	318	48	56	126	548
Percentage	58	9	10	23	100

Note: Concordance, 83% (95% CI of 80%, 86%).

Source: HercepTest[®] PMA.

APPENDIX B (cont'd)
Additional Concordance Analyses

Table 3
 HercepTest[®]/Pathway[™] 3 × 3 Concordance

Pathway [™]	HercepTest [®]			Total
	0/1+	2+	3+	
0/1+	282	14	3	299
2+	13	24	13	50
3+	4	14	83	101
Total	299	52	99	450

Note: Concordance, 86% (95% CI of 83, 90%). κ statistic (non-weighted): 0.73.

Source: Pathway[™] Package Insert.

APPENDIX C
PathVysion™ Package Insert

PathVysion™ HER-2 DNA Probe Kit
(LSI® HER-2/*neu* SpectrumOrange™ / CEP® 17 SpectrumGreen™)
Order Number 30-161060

Proprietary Name: PathVysion HER-2 DNA Probe Kit

Common or Usual Name: Fluorescence *in situ* hybridization (FISH) reagents

Intended Use

The PathVysion HER-2 DNA Probe Kit (PathVysion Kit) is designed to detect amplification of the HER-2/*neu* gene via fluorescence *in situ* hybridization (FISH) in formalin-fixed, paraffin-embedded human breast cancer tissue specimens. Results from the PathVysion Kit are intended for use as an adjunct to existing clinical and pathologic information currently used as prognostic factors in stage II, node-positive breast cancer patients. The PathVysion Kit is further indicated as an aid to predict disease-free and overall survival in patients with stage II, node positive breast cancer treated with adjuvant cyclophosphamide, doxorubicin, and 5-fluorouracil (CAF) chemotherapy.

Warning:

The Vysis PathVysion Kit is not intended for use to screen for or diagnose breast cancer. It is intended to be used as an adjunct to other prognostic factors currently used to predict disease-free and overall survival in stage II, node-positive breast cancer patients. In making decisions regarding adjuvant CAF treatment, all other available clinical information should also be taken into consideration, such as tumor size, number of involved lymph nodes, and steroid receptor status. No treatment decision for stage II, node-positive breast cancer patients should be based on HER-2/*neu* gene amplification status alone. The potential risks associated with misuse of the assay, or misinterpretation of the test results would be to assign patients to receive an adjuvant therapy regimen which is either too low for optimum effectiveness, or higher than necessary, potentially exposing the patient to serious side effects and, in rare cases, death. Selected patients with breast cancers shown to lack amplification of HER-2/*neu*, may still benefit from CAF adjuvant therapy on the basis of other prognostic factors which predict poor outcome. Conversely, selected patients with breast cancers shown to contain gene amplification may not be candidates for CAF therapy because of pre-existing or intercurrent medical illnesses. The dose and schedule of cyclophosphamide, doxorubicin, and 5-fluorouracil in the CAF regimen have not been standardized. Vysis will provide training in specimen preparation, assay procedure, and interpretation of FISH testing of the Her-2 gene for inexperienced users.

Summary and Explanation

Among all cancers in the U.S., breast cancer is expected to be the most common cancer (32% / 182,000) in women and to be the second most common cause of death from cancer (18% / 46,000) in 1995 [1]. After surgery, breast cancers with positive axillary nodes, which account for 30% of all breast cancers [2], are associated with a shorter disease-free survival [3,4] and a shorter overall survival [5] than node negative breast cancers. It has been generally accepted that patients with breast cancer and positive axillary nodes at diagnosis, should be offered adjuvant systemic treatment.

Amplification or overexpression of the HER-2/*neu* gene has been shown to be an indicator of poor prognosis in node-positive breast cancer [6-10]. In one study, the prognostic value of HER-2/*neu* appears to be stronger among patients treated with chemotherapy [7]. However, in predicting disease-free and overall survival in individual patients, other established prognostic factors such as tumor size, number of positive lymph nodes, and steroid receptor status must also be taken into consideration.

The fluorescence *in situ* hybridization (FISH) technique has been used to detect HER-2/*neu* gene amplification in human breast carcinoma cell lines in both interphase and metaphase cells [11-14]. FISH appears to be an alternative technique capable of overcoming many of the inherent technical and interpretative limitations of other techniques, such as immunohistochemistry [15]. For quantification of HER-2/*neu* gene amplification, FISH assesses not only the level of HER-2/*neu* gene amplification directly in the tumor cells while retaining the characteristic morphology of the tissue studied, but also the spatial distribution of oncogene copies in individual uncultured primary breast carcinomas.

The LSI HER-2/*neu* DNA probe is a 190 Kb SpectrumOrange directly labeled fluorescent DNA probe specific for the HER-2/*neu* gene locus (17q11.2-q12). The CEP 17 DNA probe is a 5.4 Kb SpectrumGreen directly labeled fluorescent DNA probe specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17 (17p11.1-q11.1). The probes are pre-mixed and pre-denatured in hybridization buffer for ease of use. The assay is rapid, non-radioactive, requires little tumor material, and is capable of detecting as few as 2 to 8 copies of the oncogene.

Reagents and Instruments

Materials Provided

This kit contains sufficient reagents to process approximately 20 assays. An assay is defined as one 22 mm x 22 mm target area.

- 1) LSI HER-2/*neu* SpectrumOrange (low copy number *E. coli* vector) / CEP 17 SpectrumGreen DNA Probe (*E. coli* plasmid)
 - Vysis P.N.: 30-171060
 - Quantity: 200 µL
 - Storage: -20°C in the dark
 - Composition: SpectrumGreen fluorophore-labeled alpha satellite DNA probe for chromosome 17, SpectrumOrange fluorophore-labeled DNA probe for the HER-2/*neu* gene locus, and blocking DNA, pre-denatured in hybridization buffer.
- 2) DAPI Counterstain
 - Vysis P.N.: 30-804840
 - Quantity: 300 µL
 - Storage: -20°C in the dark
 - Composition: 1000 ng/mL DAPI (4,6-diamidino-2-phenylindole) in phenylenediamine dihydrochloride, glycerol, and buffer
- 3) NP-40
 - Vysis P.N.: 30-804820
 - Quantity: 4 mL (2 vials)
 - Storage: -20 to 25°C
 - Composition: NP-40
- 4) 20X SSC salts
 - Vysis P.N.: 30-805850
 - Quantity: 66 g for up to 250 mL of 20X SSC solution
 - Storage: -20 to 25°C
 - Composition: sodium chloride and sodium citrate

Note: Material Safety Data Sheets (MSDS) for all reagents provided in the kits are available upon request from the Vysis Technical Service Department.

Storage and Handling

Store the unopened PathVysion Kit as a unit at -20°C protected from light and humidity. The 20X SSC salts and NP-40 may be stored separately at room temperature. Expiration dates for each of the unopened components are indicated on the individual component labels. These storage conditions apply to both opened and unopened components.

Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

Materials Required but Not Provided**Laboratory Reagents**

- ProbeChek HER-2/*neu* Normal Control Slides (Normal Signal Ratio) Order No. 30-805093
- Formalin-fixed, paraffin-embedded, cultured human cell line (normal LSI HER-2/*neu*:CEP 17 ratio) applied to glass microscope slides Quantity: 5 slides. Store the control slides at 15-30°C in a sealed container with desiccant to protect them from humidity.
- ProbeChek HER-2/*neu* Cutoff Control Slides (Weakly Amplified Signal Ratio) Order No. 30-805042. Formalin-fixed, paraffin-embedded, cultured human cell line (low level HER-2/*neu* amplification) applied to glass microscope slides Quantity: 5 slides. Store the control slides at 15-30°C in a sealed container with desiccant to protect them from humidity.
- Paraffin Pretreatment Reagent Kit (Vysis Cat. # 32-801200), which includes:
 - Pretreatment Solution (NaSCN) Quantity: 5 x 50 mL
 - Protease (Pepsin (2500-3000 units/mg)) Quantity: 5 x 25 mg
 - Protease Buffer (NaCl solution, pH 2) Quantity: 5 x 50 mL
 - Wash Buffer (2X SSC, pH 7) Quantity: 2 x 250 mL
- Neutral buffered formalin solution (4% formaldehyde in PBS)
- Hemo-De clearing agent (Fisher Product No. 15-182-507A)
- Hematoxylin and eosin (H & E)
- Immersion oil appropriate fluorescence microscopy. Store at room temperature.
- Ultra-pure, formamide. Store at 4°C for up to one month from delivery (See manufacturer's recommendations for detailed information).
- Ethanol (100%). Store at room temperature.
- Concentrated (12N) HCl
- 1N NaOH
- Purified water (distilled or deionized or Milli-Q). Store at room temperature.
- Rubber cement
- Drierite and Nitrogen gas

Laboratory Equipment

- Pre-cleaned silanized or positively charged glass microscope slides
- Slide warmer (45 - 50°C)
- 22 mm x 22 mm glass coverslips
- Microtiter pipettor (1-10 µL) and sterile tips
- Polypropylene microcentrifuge tubes (0.5 or 1.5 mL)
- Timer
- Microtome
- Magnetic stirrer
- Vortex mixer
- Microcentrifuge
- Graduated cylinder
- Water baths (37±1°C, 72±1°C, and 80±1°C)
- Protein-free water bath (40°C)
- Air incubators (37°C and 56°C)
- Diamond-tipped scribe
- Humidified hybridization chamber
- Forceps
- Disposable syringe (5 mL)
- Coplin jars (6) Suggested type: Wheaton Product No. 900620 vertical staining jar
- Fluorescent microscope equipped with recommended filters (see next section)
- pH meter and pH paper
- Calibrated thermometer
- Microscope slide box with lid
- 0.45 µm pore filtration unit

Microscope Equipment and Accessories

Microscope: An epi-illumination fluorescence microscope is required for viewing the hybridization results. *If an existing fluorescence microscope is available, it should be checked to be sure it is operating properly to ensure optimum viewing of fluorescence in situ hybridization assay specimens.* A microscope used with general DNA stains such as DAPI, Propidium Iodide, and quinacrine may not function adequately for FISH assays. Routine microscope cleaning and periodic "tune-ups" by the manufacturer's technical representative are advisable.

Note: Often, a presumed failure of reagents in an *in situ* assay may actually indicate that a malfunctioning or sub-optimal fluorescence microscope or incorrect filter set is being used to view a successful hybridization assay.

Excitation Light Source: A 100 watt mercury lamp with life maximum of about 200 hours is the recommended excitation source. Record the number of hours that the bulb has been used and replace the bulb before it exceeds the rated time. Ensure that the lamp is properly aligned.

Objectives: Use oil immersion fluorescence objectives with numeric apertures ≥ 0.75 when using a microscope with a 100 watt mercury lamp. A 40X objective, in conjunction with 10X eyepieces, is suitable for scanning. For FISH analysis, satisfactory results can be obtained with a 63X or 100X oil immersion achromat type objective.

Immersion Oil: The immersion oil used with oil immersion objectives should be one formulated for low auto fluorescence and specifically for use in fluorescence microscopy.

Filters: Multi-bandpass fluorescence microscope filter sets optimized for use with the CEP and LSI DNA probe kits are available from Vysis for most microscope models. The recommended filter sets for the PathVysion Kit are the DAPI/9-Orange dual bandpass, DAPI/Green dual bandpass and DAPI/Green/Orange triple bandpass. Hybridization of the LSI HER-2/*neu* and CEP 17 probes to their target regions is marked by orange and green fluorescence, respectively. All of the other DNA will fluoresce blue with the DAPI stain.

Preparation of Working Reagents**20X SSC (3M sodium chloride, 0.3M sodium citrate, pH 5.3)**

To prepare 20X SSC pH 5.3, add together:

66 g	20X SSC
200 mL	purified water
250 mL	final volume

Mix thoroughly. Measure pH at room temperature with a pH meter. Adjust pH to 5.3 with concentrated HCl. Bring the total volume to 250 mL with purified water. Filter through a 0.45 µm pore filtration unit. Store at room temperature for up to 6 months.

Denaturing Solution (70% formamide / 2X SSC, pH 7.0-8.0)

To prepare denaturing solution, add together:

49 mL	formamide
7 mL	20X SSC, pH 5.3
14 mL	purified water
70 mL	final volume

Mix thoroughly. Measure pH at room temperature using a pH meter with glass pH electrode to verify that the pH is between 7.0 - 8.0. This solution can be used for up to one week. Check pH prior to each use. Store at 2 - 8°C in a tightly capped container when not in use.

Ethanol Solutions

Prepare v/v dilutions of 70%, 85%, and 100% using 100% ethanol and purified water. Dilutions may be used for one week unless evaporation occurs or the solution becomes diluted due to excessive use. Store at room temperature in tightly capped containers when not in use.

Post-Hybridization Wash Buffer (2X SSC/0.3% NP-40)

To prepare, add together:

100 mL	20X SSC, pH 5.3
847 mL	Purified water
3 mL	NP-40
1000 mL	Final Volume

Mix thoroughly. Measure pH at room temperature using a pH meter. Adjust pH to 7.0 - 7.5 with 1N NaOH. Adjust volume to 1 liter with purified water. Filter through 0.45 µm pore filtration unit. Discard used solution at the end of each day. Store unused solution at room temperature for up to 6 months.

Warnings and Precautions

1. For *In Vitro* Diagnostic Use.
2. The PathVysion Kit is intended for use only on formalin fixed, paraffin-embedded breast cancer tissue; it is not intended for use on fresh or non-breast cancer tissue.
3. All biological specimens should be treated as if capable of transmitting infectious agents. The control slides provided with this kit are manufactured from human cell lines that have been fixed in 10% formalin. *Because it is often impossible to know which might be infectious, all human specimens and control slides should be treated with universal precautions. Guidelines for specimen handling are available from the U.S. Centers for Disease Control and Prevention [16].*
4. Exposure of the specimens to acids, strong bases, or extreme heat, should be avoided. Such conditions are known to damage DNA and may result in FISH assay failure.
5. Failure to follow all procedures for slide denaturation, hybridization, and detection may cause unacceptable or erroneous results.
6. To identify target areas, H & E staining should be conducted on every 10th slide of the same tissue block.
7. Hybridization conditions may be adversely affected by the use of reagents other than those provided by Vysis, Inc.
8. Proper storage of kit components is essential to ensure the labeled shelf life. Assay results may be adversely effected by kit components stored under other conditions.
9. If stored at low temperatures, 20X SSC may crystallize. If the crystals cannot be redissolved at room temperature, the solution should be discarded.
10. If any other working reagents precipitate or become cloudy, they should be discarded and fresh solutions prepared.
11. The DAPI Counterstain contains DAPI (4,6-diamidino-2-phenylindole) and 1,4-phenylenediamine.
 - DAPI is a possible mutagen based on positive genotoxic effects. Avoid inhalation, ingestion, or contact with skin.
 - 1,4-phenylenediamine is a known dermal sensitizer and a possible respiratory sensitizer. Avoid inhalation, ingestion, or contact with skin. Refer to MSDS for specific warnings.
12. Fluorophores are readily photobleached by exposure to light. To limit this degradation, handle all solutions containing fluorophores in reduced light. This includes all steps involved in handling the hybridized slide. Carry out all steps which do not require light for manipulation (incubation periods, washes, etc.) in the dark.
13. LSI HER-2/*neu* & CEP 17 DNA probe mixture contains formamide, a teratogen. Avoid contact with skin and mucous membranes.
14. Calibrated thermometers are required for measuring temperatures of solutions, water baths, and incubators.
15. Always verify the temperature of the pretreatment solution, denaturation solution and wash buffers prior to each use by measuring the temperature of the solution in the coplin jar with a calibrated thermometer.
16. All hazardous materials should be disposed of according to your local and state guidelines for hazardous disposal.

Specimen Processing and Slide Preparation

Specimen Collection and Processing

The PathVysion Kit is designed for use on formalin-fixed, paraffin-embedded tissue specimens. Tissue collections should be performed according to the laboratory's standard procedures. **Selection of tissue for PathVysion assay should be performed by the pathologist.** Exposure of the specimens to acids, strong bases, or extreme heat, should be avoided. Such conditions are known to damage DNA and may result in FISH assay failure.

Breast tissue should be prepared in sections between 4 and 6 microns thick. Formalin-fixed, paraffin-embedded tissue may be handled and stored according to the laboratory's routine procedures. To ensure optimum results from the PathVysion Kit, these methods should be consistent for all specimens analyzed. To identify target areas, H & E staining should be conducted on every 10th slide of the same tissue block.

Tissue sections should be mounted on the positive side of an organosilane-coated slide in order to minimize detachment of the tissue from the slide during FISH assay. The PathVysion Kit contains reagents sufficient for approximately 20 assays; one assay for the PathVysion Kit is defined as a 22 mm x 22 mm area. Larger specimen sections will require more than 10 μ L of probe per assay.

Slide Preparation from Formalin-Fixed, Paraffin-Embedded Tissue

The following method may be used for preparing slides from formalin-fixed, paraffin-embedded tissue specimens:

1. Cut 4-6 μ m thick paraffin sections using a microtome.
2. Float the sections in a protein-free water bath at 40°C.
3. Mount the section on the positive side of an organosilane-coated slide.
4. Allow slides to air dry.

(Start processing ProbeChek control slides here)

5. Bake slides overnight at 56°C.

Slide Pretreatment

Slides must be deparaffinized and the specimens fixed prior to assay with the PathVysion Kit. The package insert for the Vysis Paraffin Pretreatment Reagent Kit (Product No. 32-801200) contains detailed instructions. The following is a brief description of the procedure.

Deparaffinizing Slides

- Immerse slides in Hemo-De for 10 minutes at room temperature.
- Repeat twice using new Hemo-De each time.
- Dehydrate slides in 100% EtOH for 5 minutes at room temperature. Repeat.
- Air dry slides or place slides on a 45-50°C slide warmer.

Pretreating Slides

- Immerse slides in 0.2N HCl for 20 minutes.
- Immerse slides in purified water for 3 minutes.
- Immerse slides in Wash Buffer for 3 minutes.
- Immerse slides in Pretreatment Solution at 80°C for 30 minutes.
- Immerse slides in purified water for 1 minute.
- Immerse slides in Wash Buffer for 5 minutes. Repeat.

Protease Treatment

- Remove excess buffer by blotting edges of the slides on a paper towel.
- Immerse slides in Protease Solution at 37°C for 10 minutes.
- Immerse slides in Wash Buffer for 5 minutes. Repeat.
- Dry slides on a 45-50°C slide warmer for 2-5 minutes.

Fixing the Specimen

- Immerse the slides in neutral buffered formalin at room temperature for 10 minutes.
- Immerse the slides in wash buffer for 5 minutes. Repeat.
- Dry slides on a 45-50°C slide warmer for 2-5 minutes.
- Proceed with the PathVysion assay protocol.

Assay Procedure

Fluorescence In Situ Hybridization Procedure Summary

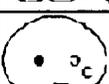
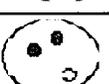
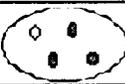
Denaturation of Specimen DNA

The timing for preparing the probe solutions should be carefully coordinated with denaturing the specimen DNA so that both will be ready for the hybridization step at the same time.

1. Prewarm the humidified hybridization chamber (an airtight container with a piece of damp blotting paper or paper towel approximately 1 in. x 3 in. taped to the side of the container) to 37°C by placing it in the 37°C incubator prior to slide preparation. Moisten the blotting paper or paper towel with water before each use of the hybridization chamber.
2. Verify that the pH of the denaturing solution is 7.0 - 8.0 at room temperature before use. Add denaturing solution to Coplin jar and place in a 72±1°C water bath for at least 30 minutes, or until the solution temperature reaches 72±1°C. Verify the solution temperature before use.
3. Mark the areas to be hybridized with a diamond-tipped scribe.
4. Denature the specimen DNA by immersing the prepared slides in the denaturing solution at 72±1°C (≤6 slides per jar) for 5 minutes. Do not denature more than 6 slides at one time per Coplin jar. *Note: Verify the solution temperature before each use.*
5. Using forceps, remove the slide(s) from the denaturing solution and immediately place into a 70% ethanol wash solution at room temperature. Agitate the slide to remove the formamide. Allow the slide(s) to stand in the ethanol wash for 1 minute.
6. Remove the slide(s) from 70% ethanol. Repeat step 5 with 85% ethanol, followed by 100% ethanol.
7. Drain the excess ethanol from the slide by touching the bottom edge of the slide to a blotter, and wipe the underside of the slide dry with a laboratory wipe.
8. Dry the slide(s) on a 45-50°C slide warmer for 2-5 minutes.

Figure 1
Dual Color Signal Counting Guide

Key :  = green probe, CEP 17
 = orange probe, LSI HER-2/neu

1		Nuclei are overlapping and all areas of both of the nuclei are not visible but signals are not in overlapping area. Count as two orange and two green in each nucleus.
2		Count as two orange signals and two green signals. One orange signal is diffuse.
3		Don't count. Nuclei are overlapping, all areas of nuclei are not visible and some signals are in overlapping area.
4		Count as two orange signals and two green signals. One orange signal is split.
5		Count as one orange signal and two green signals. One green signal is split and the orange signal is split.
6		Count as two orange signals and one green signal.
7		Count as three orange signals and one green signal.
8		Count as four orange signals and two green signals.

Quality Control

Use of Control Slides

According to good clinical laboratory practices, control slides must be run concurrently with patient slides to monitor assay performance and to assess the accuracy of signal enumeration. Control slides should be used beginning with the Slide Pretreatment procedure. Controls should be run on each day of FISH testing and with each new kit lot. Vysis ProbeChek control slides are recommended.

Slide adequacy and signal enumeration should be assessed using the criteria described above in the signal enumeration section. The criteria for slide adequacy must be satisfied and the signal enumeration results should be within the specifications on the data sheets provided with the control slides for acceptable test performance.

If control slides fail to meet the slide acceptance criteria, the assay may not have been performed properly or the PathVysion Kit component(s) may have been performed inadequately. A repeat analysis with fresh control slides and patient specimen slide(s) will be necessary. Consult the troubleshooting guide in Table 2 for probable causes and the actions needed to correct the problems.

If control slides meet the acceptance criteria but the enumeration values are outside the specified range, the enumeration may not have been performed correctly and an independent, repeat analysis of the same slide may be appropriate.

In no case should routine FISH test results be reported if assay controls fail. For clinical specimens, when interpretation of the hybridization signal is difficult and there is insufficient specimen sample for re-assay, the test is uninformative. If there are insufficient cells for analysis, the test is uninformative.

The PathVysion Kit must be tested with the ProbeChek control slides (refer to Materials Required but Not Provided). The negative control represents a specimen with a normal LSI HER-2/neu to CEP 17 signal ratio, and has a HER-2/neu to chromosome 17 copy number ratio of 1.0 to 1.2. The cutoff control represents weak HER-2/neu amplification (at or near the cutoff point) and has a HER-2/neu to chromosome 17 copy number ratio of 1.6 to 2.0. Additional cutoff (product number 30-805042) and negative (product number 30-805093) control slides are available from Vysis, Inc. Detailed descriptions of the acceptable ranges for the ratio of LSI HER-2/neu to CEP 17 are indicated on the specification data sheet that accompanies these slides.

The expected signal distribution for the normal and amplified signal ratio control slides are given in Table 1. If the results fall outside the expected range, consult the troubleshooting guide provided in Table 2.

Probe Preparation

1. Allow the probe to warm to room temperature so that the viscosity decreases sufficiently to allow accurate pipetting.
2. Vortex to mix. Centrifuge each tube for 2-3 seconds in a bench-top microcentrifuge to bring the contents to the bottom of the tube. Gently vortex again to mix.

Hybridization

1. Apply 10 µL of probe mixture to target area of slide. Immediately, place a 22 mm x 22 mm glass coverslip over the probe and allow it to spread evenly under the coverslip. Air bubbles will interfere with hybridization and should be avoided. The remaining probe solution should be refrozen immediately after use.
2. Seal coverslip with rubber cement as follows: Draw the rubber cement into a 5 mL syringe. Eject a small amount of rubber cement around the periphery of the coverslip overlapping the coverslip and the slide, thereby forming a seal around the coverslip.
3. Place slides in the pre-warmed humidified hybridization chamber. Cover the chamber with a tight lid and incubate at 37°C overnight (14-18 hours).

Post-Hybridization Washes

1. Add post-hybridization wash buffer (2X SSC/0.3% NP-40) to a Coplin jar. Prewarm the post-hybridization wash buffer by placing the Coplin jar in the 72±1°C water bath for at least 30 minutes or until solution temperature has reached 72±1°C. *Note: The temperature of the wash solution must return to 72±1°C before washing each batch.*
2. Add post-hybridization wash buffer to a second Coplin jar and place at room temperature. Discard both wash solutions after 1 day of use.
3. Remove the rubber cement seal from the first slide by gently pulling up on the sealant with forceps.
4. Immerse slide(s) in post-hybridization wash buffer at room temperature and float off coverslip.
5. After coverslip has been carefully removed, remove excess liquid by wicking off the edge of the slide and immerse slide in post-hybridization wash buffer at 72±1°C for 2 minutes (≤6 slides/jar).
6. Remove each slide from the wash bath and air dry in the dark in an upright position. (A closed drawer or a shelf inside a closed cabinet is sufficient.)
7. Apply 10 µL of DAPI counterstain to the target area of the slide and apply a glass coverslip. Store the slide(s) in the dark prior to signal enumeration.

Slide Storage

Store hybridized slides (with coverslips) at -20°C in the dark. After removing from -20°C storage, allow slide(s) to reach room temperature prior to viewing using fluorescence microscopy.

Signal Enumeration

Assessing Slide Adequacy

Evaluate slide adequacy using the following criteria:

- Probe Signal Intensity: The signal should be bright, distinct, and easily evaluable. Signals should be in either bright, compact, oval shapes or stringy, diffuse, oval shapes.
 - Background: The background should appear dark or black and relatively free of fluorescence particles or haziness.
- If any of the above features are unsatisfactory, consult the troubleshooting guide (Table 2) and process a fresh slide.

Recognition of Target Signals

Use the prescribed filter (see pg. 8). Adjust the depth of the focus, and become familiar with the size and shape of the target signals and noise (debris). Enumerate hybridization signals only among tumor cells. Tumor cells in general are larger than normal cells, lymphocytes, and epithelial cells. Identify target areas by H & E stain on every 10th slide of the same tissue block. Identify these areas on the coverslip after the FISH assay is performed.

Selection of Optimum Viewing Area and Evaluable Nuclei

Use a 25X objective to view the hybridized area and locate the target of interest (tumor cells as identified by H & E stain). Avoid areas of necrosis and where the nuclear borders are ambiguous. Skip those nuclei with signals that require subjective judgment. Skip signals with weak intensity and non-specificity, or with noisy background. Skip nuclei with insufficient counterstain to determine the nuclear border. Enumerate only those nuclei with discrete signals.

Signal Enumeration

Using a 40X objective, scan several areas of tumor cells to account for possible heterogeneity. Select an area of good nuclei distribution, avoid areas of the target where hybridization signals are weak. Using a 63X or 100X objective, begin analysis in the upper left quadrant of the selected area and, scanning from left to right, count the number of signals within the nuclear boundary of each evaluable interphase cell according to the guidelines provided below and in Figure 1.

- Focus up and down to find all of the signals present in the nucleus.
- Count two signals that are the same size and separated by a distance equal or less than the diameter of the signal as one signal.
- Do not score nuclei with no signals or with signals of only one color. Score only those nuclei with one or more FISH signals of each color.
- Record counts in a two-way table such as that shown below.

Continue this process until 60 nuclei are enumerated and analyzed.

HER-2	CEP17											Total
	0	1	2	3	4	5	6	7	8	9	10+	
0												
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11-15												
16-20												
21+												
Total												

Table 1
Signal Distribution for the ProbeChek HER-2/*neu* Normal and Amplified Signal Ratio Control Slides

Normal Ratio Control Slides						
	percentage of cells with:					
	0 signals	1 signal	2 signals	3 signals	4 signals	>4 signals
HER-2/ <i>neu</i>	0.0-2.0	8.0-12.0	27.0-37.0	43.0-62.0	1.0-3.0	1.0-3.0
CEP 17	0.0-2.0	13.0-17.0	22.0-32.0	43.0-63.0	1.0-3.0	1.0-3.0

Cutoff (Weakly Amplified Ratio) Control Slides						
	percentage of cells with:					
	0 signals	1 signal	2 signals	3 signals	4 signals	>4 signals
HER-2/ <i>neu</i>	0.0-1.0	4.0-11.0	61.0-72.0	4.0-6.0	16.0-19.0	0.0-2.0
CEP 17	0.0-1.0	85.0-93.0	23.0-29.0	0.0-1.0	0.0-3.0	0.0-1.0

Signal distribution is based on hybridization using Vysis PathVysion HER-2/*neu* probe (Cat. no. 30-171060). Refer to the Certificate of Analysis for the actual counts on individual lot numbers.

Table 2
Troubleshooting Guide

Problem	Probable Causes	Solution
o No signal or weak signals	<ul style="list-style-type: none"> o Inappropriate filter set used to view slides o Microscope not functioning properly o Improper lamps (i.e. Xenon or Tungsten) o Mercury lamp too old o Mercury lamp misaligned o Dirty and/or cracked collector lenses o Dirty or broken mirror in lamp house o Hybridization conditions inappropriate o Inappropriate post-hybridization wash temperature o Air bubbles trapped under coverslip and prevented probe access o Insufficient amount of hybridization solution for section o Inadequate protease digestion o Section overfixed o DNA loss (poor DAPI staining) 	<ul style="list-style-type: none"> o Use recommended filters o Call microscope manufacturer's technical representative o Use a mercury lamp (100 watt recommended) o Replace with a new lamp o Realign lamp o Clean and replace lens o Clean or replace mirror o Check temp. of 37±1°C incubator o Increase hybridization time to at least 14 hours o Check temp. of 72±1°C water bath o Apply coverslip by first touching the surface of the hybridization mixture o Increase amount of hybridization solution to 20 µL per section o Check temp. of 37±1°C bath o Check that pH of buffer is 2.0±0.2 o Increase digestion time, up to 60 min. o Eliminate fixation o Optimal fixation time in preparation of paraffin embedded slides is 24-48 hrs. Longer fixation times will lead to progressive degradation of morphology o Check fixation conditions
o Variation of signal intensity across tissue section	<ul style="list-style-type: none"> o Inherent in many tissue sections o Probe unevenly distributed on slide due to air bubbles under coverslip o Overfixed section 	<ul style="list-style-type: none"> o Check DAPI staining. If DAPI staining in poor areas is good, then score slide. If DAPI staining is poor in poor areas, increase fixation time o Repeat hybridization on next adjacent section or same slide and make sure no air bubbles are trapped under coverslip o Increase volume of hybridization solution to 20 µL on large tissue sections
o Noisy background	<ul style="list-style-type: none"> o Inadequate wash stringency 	<ul style="list-style-type: none"> o Check pH of 7.2-7.5 wash buffer o Check temperature of 72±1°C bath o Provide gentle agitation during wash o Increase wash time to 5 minutes o Check fixation time/conditions
o Tissue loss or tissue morphology degraded	<ul style="list-style-type: none"> o Tissue section underfixed (poor DAPI staining) o Inappropriate slides used o Improper slide baking o Over-treatment o Over-denaturation o Tissue section overfixed (cell boundaries will be distinct) o Tissue section was torn removing coverslip after hybridization 	<ul style="list-style-type: none"> o Use positively charged slides o Check temp. of 56°C oven o Check temp. of 80±1°C pretreatment o Decrease pretreatment time o Decrease protease digestion time o Check temp. of 72±1°C denaturation bath o Decrease denaturation time o Eliminate fixation step o Optimal fixation time in preparation of paraffin embedded slides is 24-48 hrs. Longer fixation times will lead to progressive degradation of morphology o Allow coverslip to soak off in wash buffer

Contact the Vysis Technical Services Department at 800-553-7042 for further assistance

Interpretation of Results

The number of LSI HER-2/*neu* and CEP 17 signals per nucleus are recorded in a two-way table. Results on enumeration of 60 interphase nuclei from tumor cells per target are reported as the ratio of average HER-2/*neu* copy number to that of CEP 17. Our clinical study found that specimens with amplification showed a LSI HER-2/*neu*:CEP 17 signal ratio of ≥2.0; normal specimens showed a ratio of <2.0.

Results at or near the cutoff point (1.8 - 2.2) should be interpreted with caution. The specimen slide should be re-enumerated by another technician to verify the results. If still in doubt, the assay should be repeated with a fresh specimen slide. If the test results are not consistent with the clinical findings, a consultation between the pathologist and the treating physician is warranted.

Reasons to Repeat the Assay

The following are situations requiring repeat assays with fresh specimen slides and the appropriate control slides. Consult the troubleshooting guide (Table 2) for probable causes and the actions needed to correct specific problems.

1. If one or both of the control slides fail to meet the slide acceptance criteria, the specimen slide results are not reliable, and the assay must be repeated.
2. If there are fewer than 60 evaluable nuclei, the test is uninformative and the assay should be repeated.
3. If, upon assessing the slide quality as described in the Signal Enumeration section, any of the aspects (signal intensity, background, or cross-hybridization) are unsatisfactory, the assay must be repeated.

Limitations

1. The PathVysion Kit has been optimized only for identifying and quantifying chromosome 17 and the HER-2/*neu* gene in interphase nuclei from formalin-fixed, paraffin-embedded human breast tissue specimens. Other types of specimens or fixatives should not be used.
2. The performance of the PathVysion Kit was validated using the procedures provided in this package insert only. Modifications to these procedures may alter the performance of the assay.
3. Performance characteristics of the PathVysion Kit have been established only for node positive patients receiving the designated regimens of CAF. Performance with other treatment regimens has not been established.
4. The clinical interpretation of any test results should be evaluated within the context of the patient's medical history and other diagnostic laboratory test results.
5. FISH assay results may not be informative if the specimen quality and/or specimen slide preparation is inadequate.
6. Technologists performing the FISH signal enumeration must be capable of visually distinguishing between the orange and green signals.

Expected Values

FISH interphase signal enumeration was performed on normal and amplified human breast tissue specimens to assess the expected ratio of LSI HER-2/*neu* to CEP 17.

FISH interphase analysis was performed on human breast tissue specimens from 433 subjects which were negative for HER-2/*neu* gene amplification by immunohistochemistry (IHC), to determine the expected values and to verify the cutoff point. Sixty nuclei were enumerated per specimen, the distribution of ratios of HER-2/*neu* to CEP 17 signals for these subjects is summarized in Table 3.

Table 3
Distribution of Ratio of HER-2/*neu* to CEP 17 Signals in Non-Amplified Breast Tissue Specimens

Statistics	Range		
	0.1-1.0	1.1-1.5	1.6-1.99
mean	0.86	1.15	1.72
S.D.	0.14	0.13	0.11
n	185	226	22

The cutoff point for assessing HER-2/*neu* gene amplification in breast tissue specimens was 2.0.

Ninety-one HER-2/*neu* amplified breast tissue specimens were also tested. The distribution of ratios of HER-2/*neu* to CEP 17 signals for these subjects is summarized in Table 4.

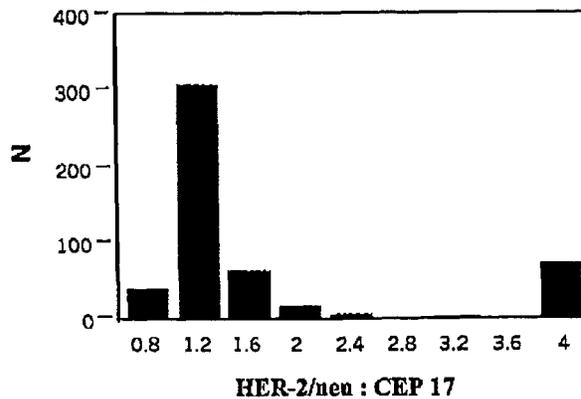
Table 4
Distribution of Ratio of HER-2/*neu* to CEP 17 Signals in Amplified Breast Tissue Specimens

Statistics	Range		
	2.0-5.0	5.1-10.0	>10.0
mean	3.35	7.39	12.77
S.D.	0.95	1.41	1.80
n	33	42	16

Establishment of Cut-off Point

In the pivotal study, the cutoff point for determining HER-2/*neu* gene amplification was determined to be 2.0, based on best fit analysis of clinical outcome of CAF treatment. Among the 433 non-amplified specimens, the largest ratio of LSI HER-2/*neu* to CEP 17 signals was 1.95, and among the 91 amplified samples, the smallest ratio of LSI HER-2/*neu* to CEP 17 signals was 2.0. This gap between the largest value among normal specimens and the smallest value among amplified specimens reduces the chance of misclassification, with 2.0 as the cutoff point. The distribution of the ratio of LSI HER-2/*neu*:CEP 17 in the 524 specimens from the study described above are shown in Figure 2.

Figure 2
Signal Distribution



Performance Characteristics
Analytical Sensitivity and Specificity

Hybridization Efficiency

On the ProbeCheck™ quality control slides, the average percentage of cells with no hybridization signal was 0.0 to 2.0%. These slides are prepared from formalin-fixed, paraffin-embedded breast cancer cell lines, and represent the best case scenario for hybridization efficiency. Thus, under these conditions, the hybridization efficiency is expected to be 98%, with <2% cells having no signal for either probe.

Analytical Sensitivity

The analytical sensitivity of the PathVysion Kit probes was tested in the reproducibility study described below. In that study, the 1.0 - 1.2 HER-2/*neu* : CEP 17 ratio specimen was estimated with a mean of 1.05 (±0.03), and the 1.6 - 2.0 HER-2/*neu* CEP 17 ratio specimen was estimated with a mean of 1.81 (±0.08). The upper 95% CI was 1.11 for the 1.0 - 1.2 ratio specimen and the lower 95% CI for the 1.6 - 2.0 specimen was 1.65. Thus, the limit of detection for the PathVysion Kit in interphase cells is estimated to be a ratio of 1.5.

Analytical Specificity

Locus specificity studies were performed with metaphase spreads according to standard Vysis QC protocols. A total of 254 metaphase spreads were examined sequentially by G-banding to identify chromosome 17, and the HER-2/*neu* gene locus, followed by FISH. No cross-hybridization to other chromosome loci was observed in any of the 254 cells examined; hybridization was limited to the intended target regions of the two probes.

Stringency studies were also performed, according to standard Vysis protocols, on formalin-fixed, paraffin-embedded tissue specimens to determine the optimum denaturation time and temperature; hybridization time and temperature; post-hybridization wash time and temperature; and post-hybridization wash buffer composition. For the denaturation step, three temperatures (65°C, 73°C, and 80°C) were tested for 2 minutes, 5 minutes and 8 minutes each. The results showed no statistical difference in the overall rating among all denaturation temperatures and durations; all combinations passed the quality evaluation. Stringency of the hybridization step was tested in two parts: first, hybridizations were conducted at 5 different temperatures (27°C, 32°C, 37°C, 42°C, and 47°C) for 18 hours, then for 5 different durations (10 hr, 14 hr, 18 hr, 22 hr, and 26 hr) at the recommended temperature (37°C). Hybridization was significantly affected by both hybridization temperature and time, with hybridizations at 37°C for 18 hours showing the highest overall quality ratings. The post-hybridization wash step was tested in a similar manner; first assays were conducted at 5 different temperatures (69°C, 71°C, 73°C, 76°C, and 80°C), then for different durations, ranging from 2 to 8 minutes at 73°C. Wash temperature was a significant factor, with 73°C resulting in the highest ratings. Wash times between 2 and 5 minutes all produced acceptable results, but increasing the wash time to 8 minutes significantly lowered the overall quality ratings in some samples. The wash buffer composition was also analyzed to determine the effect on signal intensity and probe specificity. Increasing the salt concentration from 0.4X SSC to 2X SSC increased the signal intensity, but did not appear to compromise the probe specificity. Thus, a wash buffer composition of 2X SSC / 0.3% NP-40 is recommended.

Reproducibility

To assess the reproducibility of the HER-2/*neu* and CEP 17 assay, analyses for the ratio of HER-2/*neu* to CEP 17 were assessed for inter-site, inter-lot, inter-day, and inter-observer reproducibility on control slides with differing levels of HER-2/*neu* gene amplification. Four specimens consisting of formalin-fixed, paraffin-embedded tissue from human breast tumor cell lines with normal (1.0-1.2) and amplified (1.6-2.0, 3-5, 7-11) ratios of HER-2/*neu* to CEP 17 were evaluated for HER-2/*neu* and CEP 17 according to the instructions for signal enumeration in the package insert. The overall hybridization success rate was 98.3% (118/120) on the first try. Hybridization of the two replacement slides was successful.

Using ANOVA, statistically significant variations were observed between observers, which reflects the subjectivity in signal interpretation and enumeration. No statistically significant variations were observed in any of the other study parameters. The mean, standard deviation, and percent CV of the observed ratios of HER-2/*neu* to CEP 17 are shown in Tables 5-8.

Table 5
Site-to-Site Reproducibility

Ratio of HER-2/ <i>neu</i> to CEP 17	Statistics	Site #1	Site #2	Site #3
1.0-1.2	Mean	1.08	1.01	1.07
	S.D.	0.03	0.04	0.07
	C.V.(%)	2.66	3.58	6.77
	n	6	6	6
1.6-2.0	Mean	1.81	1.71	1.78
	S.D.	0.05	0.05	0.19
	C.V.(%)	2.88	2.78	10.50
	n	6	6	6
3.0-5.0	Mean	4.39	3.65	4.49
	S.D.	0.22	0.18	0.79
	C.V.(%)	4.99	4.93	17.64
	n	6	6	6
7.0-11	Mean	7.21	8.26	8.23
	S.D.	0.15	0.83	0.87
	C.V.(%)	2.07	10.10	10.55
	n	6	6	6

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

Table 6
Lot-to-Lot Reproducibility

Ratio of HER-2/ <i>neu</i> to CEP 17	Statistics	Lot #1	Lot #2	Lot #3	Lot #4
1.0-1.2	Mean	1.05	1.07	1.02	1.04
	S.D.	0.07	0.06	0.03	0.05
	C.V.(%)	6.48	6.06	3.21	4.87
	n	6	6	6	6
1.6-2.0	Mean	1.78	1.77	1.77	1.75
	S.D.	0.10	0.13	0.15	0.09
	C.V.(%)	5.65	7.49	8.54	5.07
	n	6	6	6	6
3.0-5.0	Mean	4.08	3.92	4.57	4.14
	S.D.	0.44	0.34	0.96	0.40
	C.V.(%)	10.78	8.74	20.92	9.56
	n	6	6	6	6
7.0-11	Mean	7.57	7.72	7.89	8.33
	S.D.	0.69	0.72	0.88	1.06
	C.V.(%)	8.97	9.36	11.16	12.68
	n	6	6	6	6

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

Table 7
Day-to-Day Reproducibility

Ratio of HER-2/1neu to CEP 17	Statistics	Assay Day #1	Assay Day #2	Assay Day #3	Assay Day #4
1.0-1.2	Mean	1.06	1.07	1.02	1.04
	S.D.	0.06	0.07	0.05	0.04
	C.V.(%)	5.65	6.61	4.58	4.03
	n	6	6	6	6
1.6-2.0	Mean	1.76	1.77	1.77	1.77
	S.D.	0.17	0.14	0.08	0.10
	C.V.(%)	9.62	7.99	4.31	5.65
	n	6	6	6	6
3.0-5.0	Mean	4.24	4.41	4.10	3.89
	S.D.	0.48	0.97	0.36	0.38
	C.V.(%)	11.25	21.56	8.89	9.71
	n	6	6	6	6
7.0-11	Mean	7.91	8.01	7.72	7.97
	S.D.	1.11	0.90	0.57	0.89
	C.V.(%)	13.99	11.23	7.39	11.20
	n	6	6	6	6

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

Table 8
Observer-to-Observer Reproducibility

Ratio of HER-2/1neu to CEP 17	Statistics	Observer #1	Observer #2
1.0-1.2	Mean	1.06	1.04
	S.D.	0.07	0.03
	C.V.(%)	7.00	2.85
	n	12	12
1.6-2.0	Mean	1.71	1.82
	S.D.	0.10	0.11
	C.V.(%)	6.01	6.20
	n	12	12
3.0-5.0	Mean	4.05	4.31
	S.D.	0.44	0.73
	C.V.(%)	10.80	16.84
	n	12	12
7.0-11	Mean	7.52	8.28
	S.D.	0.49	0.95
	C.V.(%)	6.55	11.44
	n	12	12

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

Assay Portability

A five-center, blinded, randomized, comparative study using formalin-fixed, paraffin-embedded human breast cancer specimens was conducted to assess assay portability. Study specimens consisted of formalin-fixed, paraffin-embedded human breast cancer tissue sections with varying levels of HER-2/1neu gene amplification. The specimens included one normal (no amplification), two with low level, and one with moderate level HER-2/1neu gene amplification, as determined by FISH. In this study, 100% of the specimens assayed yielded interpretable results on the first try.

Day-to-Day Reproducibility

The results of this study also demonstrated that the PathVysion assay is reproducible from day to day. Table 9 shows that the mean observed ratios of LSI HER-2/1neu to CEP 17 for the three assay days varied within a narrow range, as evidenced by the low S.D.'s and C.V.'s.

Table 9
Summary Statistics of LSI HER-2/1neu to CEP 17 by Assay Day

Expected Ratio	Statistics	Assay Day #1	Assay Day #2	Assay Day #3	P-value
1.0-1.2	Mean	1.01	1.05	1.04	0.6395
	S.D.	0.08	0.10	0.05	
	C.V.(%)	7.92	9.52	4.81	
	n	5	5	5	
2.1-2.8	Mean	2.53	2.42	2.42	0.7623
	S.D.	0.11	0.28	0.39	
	C.V.(%)	4.34	11.57	16.12	
	n	5	5	5	
2.5-3.5	Mean	3.17	2.81	3.03	0.5813
	S.D.	0.27	0.30	0.30	
	C.V.(%)	8.52	10.07	9.90	
	n	5	5	5	
5.0-7.0	Mean	5.66	5.20	5.21	0.9452
	S.D.	0.29	0.25	0.42	
	C.V.(%)	5.12	4.46	7.47	
	n	5	5	5	

Site-to-Site Reproducibility

Table 10 shows that the mean observed ratios of LSI HER-2/1neu to CEP 17 for the five study sites varied within a narrow range, as evidenced by the low S.D.'s and C.V.'s.

Table 10
Site-to-Site Reproducibility

Expected Ratio	Statistics	Site #1	Site #2	Site #3	Site #4	Site #5	P-value
1.0-1.2	Mean	1.00	1.15	1.01	1.04	0.98	0.0032
	S.D.	0.03	0.06	0.06	0.02	0.02	
	C.V.(%)	3.00	5.22	5.94	1.92	2.04	
	n	3	3	3	3	3	
2.1-2.8	Mean	2.39	2.45	2.55	2.26	2.65	0.4919
	S.D.	0.15	0.24	0.46	0.18	0.20	
	C.V.(%)	6.28	9.80	18.04	7.95	7.55	
	n	3	3	3	3	3	
2.5-3.5	Mean	3.00	3.09	3.41	2.73	3.08	0.0269
	S.D.	0.16	0.38	0.12	0.08	0.12	
	C.V.(%)	5.33	12.30	3.52	2.93	3.90	
	n	3	3	3	3	3	
5.0-7.0	Mean	5.41	5.19	5.89	5.73	5.91	<0.0001
	S.D.	0.07	0.21	0.07	0.08	0.05	
	C.V.(%)	1.29	4.05	1.19	1.40	0.85	
	n	3	3	3	3	3	

The summary of assay variations for all five are presented in Table 11. The standard deviation (S.D.) and the coefficient of variation (C.V.) were small and relatively stable across all ratios of LSI HER-2/neu to CEP 17.

Table 11
Summary of Site-to-Site Reproducibility

Ratio of HER-2/neu to CEP 17	Mean	Standard Deviation	C.V. (%)	N
1.0-1.2	1.04	0.07	6.73	15
2.1-2.8	2.46	0.27	10.96	15
2.5-3.5	3.06	0.28	9.15	15
5.0-7.0	5.63	0.30	5.33	15

This study had a 100% (60/60) hybridization success rate, validating the ease of use of the PathVysion HER-2 Kit.

Clinical Study

The interaction between HER-2/neu gene amplification and dose of CAF was evaluated in a retrospective analysis of a single randomized clinical trial, CALGB 8869. This was a large, prospective, randomized trial in stage II, node-positive breast cancer patients that evaluated three different doses of adjuvant CAF chemotherapy: a high dose (cyclophosphamide at 600 mg/m², doxorubicin at 60 mg/m², and 5-fluorouracil at 600 mg/m² for four cycles), a moderate dose (cyclophosphamide at 400 mg/m², doxorubicin at 40 mg/m², and 5-fluorouracil at 400 mg/m² for six cycles), or a low dose (cyclophosphamide at 300 mg/m², doxorubicin at 30 mg/m², and 5-fluorouracil at 300 mg/m² for four cycles). Doses were administered on a 28-day cycle.

Archived tissue specimens from 572 patients, randomly selected from the original study population, were included for analysis by FISH assay with DNA probe. The objectives of this study were to determine whether amplification of the HER-2/neu gene provides statistically significant and independent prognostic information pertaining to disease-free survival and overall survival in stage II, node positive breast cancer patients receiving adjuvant CAF therapy; and to explore the relationship between HER-2/neu gene amplification and clinical data, including such factors as tumor grade and steroid receptor status.

Among these 572 tumor specimens, 45 were excluded due to FISH assay failures, and 3 were duplicate assays. This left 524 cases for analysis. Using the Vysis PathVysion DNA Probe Kit, HER-2/neu gene amplification was defined as >2 (i.e., the ratio of average HER-2/neu to average CEP 17 signals with 60 nuclei counted). A total of 433 patient samples were found to be HER-2/neu-negative and 91 HER-2/neu-positive.

The table below lists the several baseline characteristics of the 524 patients whose archived tumor specimens were selected for evaluation by this assay, as well as, details of the adjuvant treatments received on the original CALGB 8869 study.

Table 12
Comparison of Patient Characteristics at Baseline and Details of Adjuvant Treatment

	HER-2/neu amplification* n=91	No HER-2/neu amplification* n=433
Age		
<40	17.6	14.5
40-50	39.6	40.0
>50	42.9	48.5
Menopausal	46.2	39.3
Peri/Postmenopausal	53.8	60.5
Tumor size		
≤ 2 cm	31.9	37.2
>2 - 5	57.1	58.4
>5	9.9	3.9
unknown	1	0.5
Positive nodes		
1	59.3	55.9
4-9	27.5	34.9
10	13.2	9.2
ER (+)	49.5	71.4
PR(+)	35.2	61.7
ER (+) or PR (+)	60.4	77.8
CAF dose regimen received		
High	33.0	34.4
Moderate	36.1	31.4
Low	33.0	34.2

*percent of patients

The results of analysis with Cox proportional hazard model for disease-free survival using FISH measurement of HER-2/neu gene amplification showed a statistically significant interaction between HER-2/neu gene amplification and the CAF dose regimen received (p=0.033, likelihood test, see Table 13). Similarly, the results of Cox proportional hazard model for overall survival also showed a statistically significant interaction between HER-2/neu gene amplification and the CAF dose regimen received (p=0.028, likelihood test, see Table 13)

Table 13
Cox Proportional Hazard Model Showing Likelihood-Ratio Tests for Disease-free and Overall Survival

Source	Disease-Free Survival			Overall Survival		
	DF	ChiSq	P value	DF	ChiSq	P value
CAF	2	5.56	0.06	2	4.57	0.10
Square root # positive nodes	1	72.87	0.0000	1	56.32	0.0000
Tumor > 2 cm	1	13.77	0.0002	1	12.03	0.0003
Pre-menopausal	1	1.96	0.16	1	0.10	0.76
HER-2 ratio	1	10.05	0.0015	1	10.52	0.0012
HER-2 ratio interaction of CAF dose	2	6.84	0.033	2	7.13	0.028

Disease-free survival probabilities (Table 14, Figure 3a) are comparable among the three dose groups of patients with HER-2/neu-negative tumors. For example, at 7 years post-randomization the estimated disease-free survival probabilities are 55%, 63%, and 61% for low (L), moderate (M), and high (H) CAF dose groups, respectively. The dose effect is greater for patients with HER-2/neu-positive tumors (Table 14, Figure 3b), with disease-free survival at 7 years of 36%, 44%, and 66% for L, M, and H CAF dose groups, respectively. The corresponding figures for overall survival at 7 years (Table 15, Figure 3c) have a similar relationship: 64%, 73%, and 70% for patients with HER-2/neu-negative tumors, and 48%, 50%, and 76% for patients with HER-2/neu-positive tumors, again for L, M, and H CAF dose groups, respectively (Table 15, Figure 3d).

Table 14
Disease-free Survival Probabilities

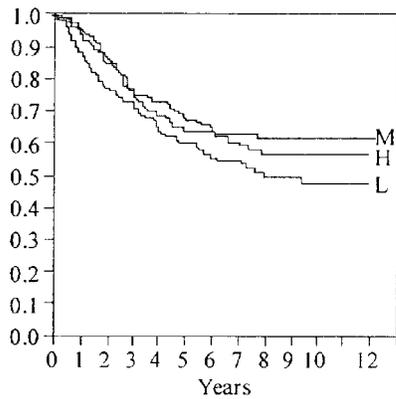
CAF Dose	HER-2/neu negative	HER-2/neu positive
Low	55%	36%
Moderate	63%	44%
High	61%	66%

Table 15
Overall Survival Probabilities

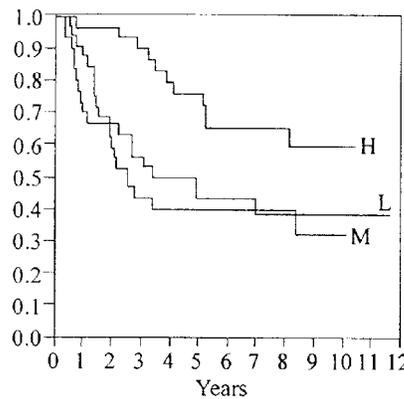
CAF Dose	HER-2/neu negative	HER-2/neu positive
Low	64%	48%
Moderate	75%	50%
High	70%	78%

Figure 3
Disease-free (a, b) and overall (c, d) survival for patients with HER-2/neu-negative (a, c) and positive (b, d) tumors for the three CAF dose groups, H, M, and L*

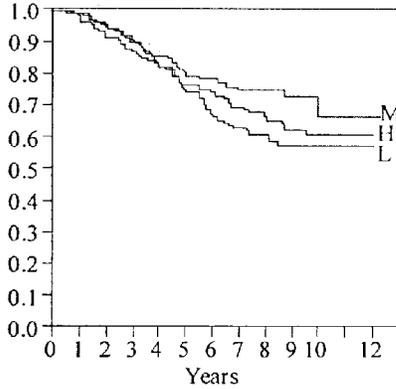
(a) Disease-free survival for HER-2/neu- negative



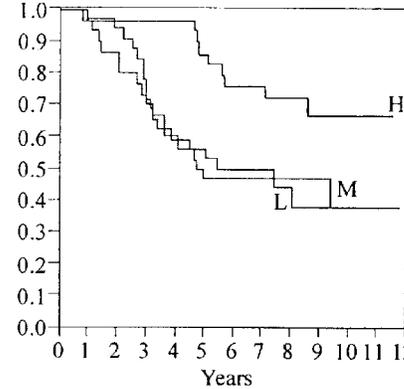
(b) Disease-free survival for HER-2/neu -positive



(c) Overall survival for HER-2/neu-negative



(d) Overall survival for HER-2/neu-positive



*HER-2/neu positivity means HER/CEP ≥ 2 . Sample sizes in (a, c) are 149, 136, and 148 (for H, M, and L) and in (b, d) are 30, 31, and 30. The significance levels for the HER-2/neu by CAF interaction from the proportional hazards models (Table 13) are 0.033 for disease-free survival—(a) vs. (b)—and 0.028 for overall survival—(c) vs. (d).

FISH analysis of the study specimens showed that there was a significant dose-response effect of adjuvant chemotherapy with CAF in patients with HER-2/neu gene amplification, but not in patients with no or minimal HER-2/neu amplification. This association was found in both disease-free and overall survival. In addition, this study found no correlation between HER-2/neu copy number, as assessed by FISH, and patient age, menopausal status, tumor size or the number of positive nodes. A statistically significant negative correlation was observed between HER-2/neu copy number and both estrogen (ER) and progesterone (PR) receptor status.

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Vysis direct label fluorescence probes and methods for use are covered by European Patent #EP 0 549 709 B1 applicable in the European countries: Italy, France United Kingdom and Germany.

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PathVysion HER-2 DNA Probe Kit Package Insert
Product Number 30-161060

30-608377 Rev D 2/2000

APPENDIX D

Evaluation of Probe Volume and Coverslip Effects

The PathVysion™ package labeling specifies that tissue sections be treated with 10 µL of probe and covered by a 22 × 22 mm coverslip. Many of the tissue sections used for the assessments in this sBLA were excessively large and could not be covered by a 22 × 22 mm coverslip.

Both LabCorp and USC elected to use larger coverslips and an increased probe volume for samples that could not be adequately encompassed by a 22 × 22 mm coverslip. Coverslips of 22 × 30 mm and 24 × 40 mm were used. An increase in probe volume (from 10 µL to either 20, 25, or 35 µL) was inconsistently employed at LabCorp (due to technician error), but was consistently employed at USC.

Both laboratories performed validation experiments to evaluate the potential role of probe volume and coverslip size in assessing FISH scores.

USC

At USC, 27 random specimens were identified that were originally scored with a larger than standard coverslip and increased probe volume. These samples were then re-assayed with a standard 22 × 22 mm coverslip and 10 µL of probe. As can be seen from Table 1, there were no significant differences in FISH scores based on coverslip size.

APPENDIX D (cont'd)
Evaluation of Probe Volume and Coverslip Effects

Table 1

Comparison of FISH Results Obtained with Large (22 × 30 mm or 24 × 40 mm)
and Standard-Sized (22 × 22 mm) Coverslips

Case No.	Original Coverslip Size	Original Ratio	Validation Run Coverslip Size	Ratio	
				Reader 1	Reader 2
1	24 × 40 mm	6.38	22 × 22 mm	6.05	6.89
2	24 × 40 mm	1.05	22 × 22 mm	1.16	1.25
3	24 × 40 mm	2.23	22 × 22 mm	2.98	3.00
4	24 × 40 mm	4.13	22 × 22 mm	5.09	4.35
5	24 × 40 mm	3.04	22 × 22 mm	3.49	3.61
6	24 × 40 mm	0.98	22 × 22 mm	1.02	0.99
7	24 × 40 mm	7.68	22 × 22 mm	6.72	7.26
8	24 × 40 mm	1.14	22 × 22 mm	1.03	1.06
9	24 × 40 mm	5.20	22 × 22 mm	4.74	5.54
10	24 × 40 mm	4.80	22 × 22 mm	4.60	4.68
11	22 × 30 mm	6.11	22 × 22 mm	6.92	7.36
12	22 × 30 mm	17.80	22 × 22 mm	12.53	11.50
13	22 × 30 mm	6.98	22 × 22 mm	5.46	5.36
14	22 × 30 mm	8.18	22 × 22 mm	8.98	8.55
15	22 × 30 mm	9.97	22 × 22 mm	6.64	6.61
16	22 × 30 mm	7.12	22 × 22 mm	6.74	6.68
17	22 × 30 mm	5.46	22 × 22 mm	5.78	5.37
18	22 × 30 mm	2.05	22 × 22 mm	2.29	2.57
19	22 × 30 mm	6.59	22 × 22 mm	7.05	5.52
20	22 × 30 mm	4.54	22 × 22 mm	3.90	3.63
21	22 × 30 mm	1.26	22 × 22 mm	1.14	1.18
22	22 × 30 mm	1.19	22 × 22 mm	1.22	1.17
23	22 × 30 mm	1.18	22 × 22 mm	1.24	1.04
24	22 × 30 mm	2.45	22 × 22 mm	2.97	2.77
25	22 × 30 mm	5.94	22 × 22 mm	5.54	5.26
26	22 × 30 mm	5.33	22 × 22 mm	5.76	6.03
27	22 × 30 mm	6.43	22 × 22 mm	5.27	5.54

APPENDIX D (cont'd)
Evaluation of Probe Volume and Coverslip Effects

LabCorp

At LabCorp, a single well-characterized “control” sample that was used to validate runs was assayed under standard conditions (22 × 22 mm, 10 μL) and then with a larger coverslip (24 × 40 mm) along with varying amounts of probe, ranging from 10 to 20 μL (Table 2). No significant differences in FISH scores were observed. The same sample was then repeatedly assayed under three different conditions of coverslip size and probe volume, with similar results (see Table 3). ANOVA results revealed F=0.106; df=9.2; p=0.90.

Table 2
 Evaluation of Coverslip Size and Probe Concentration on HER2/CEP17 Ratios

	Coverslip Size (mm) and Probe Volume				
	22 × 22 10 μL	24 × 40 10 μL	24 × 40 12 μL	24 × 40 15 μL	24 × 40 20 μL
HER2/CEP17	5.93	6.91	6.05	6.59	6.69

Table 3
 Summary of HER2/CEP17 Determinations for 22 × 22 mm
 and 24 × 40 mm Coverslips

	Coverslip Size (mm) and Probe Volume		
	22 × 22, 10 μL	24 × 40, 10 μL	24 × 40, 20 μL
Number of assays	4	6	2
HER2/CEP17 range	5.93–7.52	5.92–7.81	6.61–6.69
Mean	6.92	6.87	6.65
Standard deviation	0.72	0.74	0.06

These two independent assessments suggest that coverslip size and probe volume across the ranges used in these studies would not be expected to influence FISH scores.

APPENDIX E

Effect of Nuclei Scoring (60 vs. 40 vs. 20)

As part of the original PathVysion™ PMA, inter-laboratory reproducibility (“portability”) was evaluated. Five different laboratories (a combination of community- and university-based centers) were selected and trained in the proper performance of PathVysion™. These laboratories were then provided formalin-fixed, paraffin-embedded human breast cancer tissue sections with varying levels of HER2 gene amplification. The specimens included one normal (no amplification), two with low, and one with moderate HER2 gene amplification, as determined by FISH. The sites were fully blinded with respect to the FISH status of the supplied tissue sections. For each specimen slide, 5 nuclei were enumerated in four different target areas in accordance with the counting guide described in the PathVysion™ Package Insert (see Appendix C). This enumeration procedure was repeated until three sets of 20 nuclei were enumerated, for a total of 60 nuclei per slide.

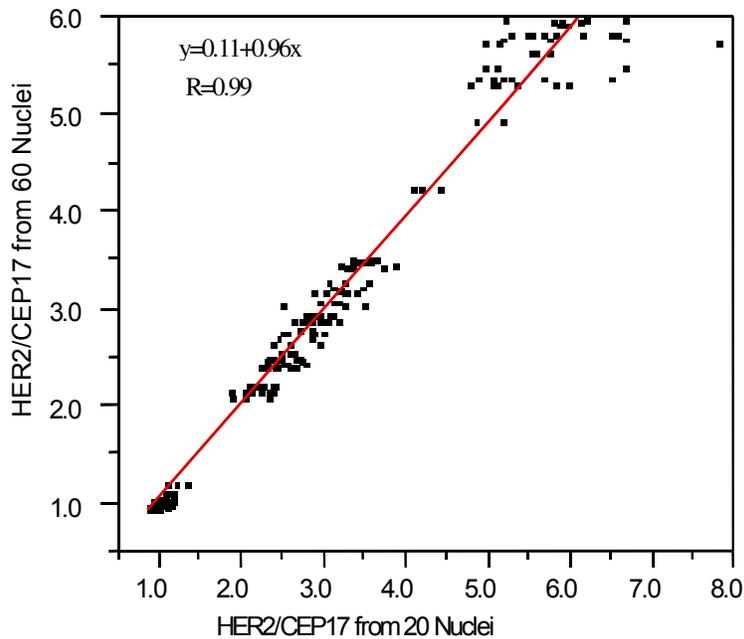
This work not only assessed inter-laboratory reproducibility but also allowed for an assessment of enumerating 20 versus 60 nuclei. The results for each of the three sets of 20 nuclei were compared with the overall results of 60 nuclei for each specimen slide. Each sample was processed in triplicate, and three sets of results were reported. The individual results for 60 nuclei are shown in Table 1, and those for 20 nuclei are reported in Table 2. The correlation between the results (60 vs. 20 nuclei enumeration) is shown in Figure 1.

Although the variation for counting 20 nuclei was greater compared with that for counting 60 nuclei, their mean ratios were similar. Figure 1 shows the correlation of the ratio of HER2 to CEP17 (correlation coefficient = 0.99) between 60 and 20 nuclei counted across the four levels of HER2 amplification and five investigation sites. The regression coefficient was 0.96, indicating a high reproducibility of signal enumeration between 60 and 20 nuclei counted. The accuracy of enumeration is greatest when counting fewer signals per cell nucleus, as indicated for the normal tissue with a ratio of 1. Very good separation in counts was achieved between the normal and low amplified

APPENDIX E (cont'd)
Effect of Nuclei Scoring (60 vs. 40 vs. 20)

tissues. The results of this comparative analysis support enumerating fewer than 60 nuclei to accurately determine the ratio of LSI HER2 to CEP17. Specifically, this analysis supports the LabCorp protocol of enumerating 40 nuclei, provided the ratio was <1.8 or >2.2, with an additional 20 nuclei enumeration (for a total of 60) for ratios between 1.8 and 2.2.

Figure 1
Correlation of Ratio of HER2 to CEP17
for Enumerating 60 versus 20 Nuclei



APPENDIX E (cont'd)
Effect of Nuclei Scoring (60 vs. 40 vs. 20)

Table 1
 Inter-Assay Portability: 60 Nuclei Enumerated per Specimen

Site		Specimen											
		Ratio of LSI HER2 to CEP17											
		1.0–1.2			2.1–2.8			2.5–3.5			5.0–7.0		
Site 1: UKMC	Observed ratio	0.98	0.98	1.03	2.51	2.44	2.23	3.18	2.88	2.94	5.37	5.39	5.51
	Summary Statistics	Mean:	1.00			2.39			3.00			5.42	
		SD:	0.03			0.15			0.16			0.07	
		CV (%):	3.00			6.28			5.33			1.29	
		n	3			3			3			3	
Site 2: RIH	Observed ratio	1.12	1.22	1.12	2.71	2.43	2.23	3.52	2.80	2.95	5.33	5.31	4.95
	Summary Statistics	Mean:	1.15			2.45			3.09			5.19	
		SD:	0.06			0.24			0.38			0.21	
		CV (%):	5.22			9.80			12.30			4.05	
		n	3			3			3			3	
Site 3: PSF	Observed ratio	0.94	1.06	1.04	2.45	2.15	2.50	3.28	3.51	3.44	5.85	5.85	5.97
	Summary Statistics	Mean:	1.01			2.36			3.41			5.89	
		SD:	0.06			0.19			0.12			0.07	
		CV (%):	5.94			8.05			3.52			1.19	
		n	3			3			3			3	
Site 4: UMC/UF	Observed ratio	1.05	1.01	1.05	2.46	2.22	2.09	2.78	2.77	2.65	5.79	5.63	5.76
	Summary Statistics	Mean:	1.04			2.26			2.73			5.73	
		SD:	0.02			0.18			0.08			0.08	
		CV (%):	1.92			7.96			2.93			1.40	
		N	3			3			3			3	
Site 5: MHMC	Observed ratio	0.95	1.00	0.97	2.57	2.88	2.50	3.10	2.95	3.19	5.95	5.85	5.92
	Summary Statistics	Mean:	0.98			2.65			3.08			5.91	
		SD:	0.02			0.20			0.12			0.05	
		CV (%):	2.04			7.55			3.90			0.85	
		n	3			3			3			3	

APPENDIX E (cont'd)
Effect of Nuclei Scoring (60 vs. 40 vs. 20)

Table 2
 Inter-Assay Portability: 20 Nuclei Enumerated per Specimen

Site			Specimen											
			Ratio of LSI HER2 to CEP17											
			1.0–1.2			2.1–2.8			2.5–3.5			5.0–7.0		
Site 1: UKMC	Observed ratio		1.11	0.89	0.94	2.47	2.38	2.71	3.40	2.89	3.26	5.68	5.29	5.17
			1.10	0.92	0.95	2.44	2.30	2.58	2.86	2.74	3.06	5.06	6.50	4.89
			1.06	1.11	0.93	2.17	2.42	2.10	2.89	2.86	3.08	4.98	5.11	6.67
	Summary Statistics	Mean:	1.00	2.40			3.01			5.48				
		SD:	0.09	0.19			0.21			0.66				
CV (%):		9.00	7.92			6.98			12.04					
n		9	9			9			9					
Site 2: RIH	Observed ratio		1.12	1.18	1.08	2.84	2.44	2.84	3.62	3.63	3.59	4.79	5.97	5.35
			1.11	1.22	1.35	2.42	2.22	2.68	2.71	2.84	2.86	5.11	5.82	5.05
			1.07	1.12	1.18	2.22	2.26	2.22	2.95	3.05	2.85	5.17	4.84	4.84
	Summary Statistics	Mean:	1.16	2.46			3.09			5.22				
		SD:	0.09	0.26			0.35			0.43				
CV (%):		7.76	10.57			11.33			8.24					
n		9	9			9			9					
Site 3: PSF	Observed ratio		0.93	0.89	1.00	2.31	2.76	2.31	3.08	3.53	3.26	6.57	5.82	5.29
			1.08	1.08	1.00	1.87	2.33	2.25	3.59	3.50	3.44	6.48	5.48	5.68
			1.00	1.07	1.06	2.31	2.41	2.74	3.32	3.72	3.30	6.677	5.23	6.22
	Summary Statistics	Mean:	1.01	2.37			3.41			5.94				
		SD:	0.07	0.27			0.20			0.56				
CV (%):		6.93	11.39			5.87			9.43					
n		9	9			9			9					
	Observed ratio		1.18	1.00	0.97	2.58	2.29	2.52	2.56	2.87	2.98	5.76	5.19	6.67
			0.95	0.98	1.14	2.36	2.13	2.21	2.90	2.89	2.52	5.59	5.75	5.55
			1.02	1.00	1.16	2.34	2.04	1.9	2.97	2.59	2.38	4.98	5.14	7.79

APPENDIX E (cont'd)
Effect of Nuclei Scoring (60 vs. 40 vs. 20)

Table 2 (cont'd)
 Inter-Assay Portability: 20 Nuclei Enumerated per Specimen

Site			Specimen											
			Ratio of LSI HER2 to CEP17											
			1.0–1.2			2.1–2.8			2.5–3.5			5.0–7.0		
Site 4: UMC/UF	Summary Statistics	Mean:	1.04			2.26			2.74			5.82		
		SD:	0.09			0.22			0.23			0.89		
		CV (%):	8.65			9.73			8.39			15.29		
		N	9			9			9			9		
Site 5: MHMC	Observed ratio		1.00	0.91	0.95	2.60	2.48	2.64	3.14	3.19	2.98	5.81	6.14	5.92
			0.97	1.05	0.97	2.63	2.83	3.17	3.12	2.79	2.95	6.17	5.85	5.56
			0.91	1.00	1.00	2.65	2.50	2.36	3.25	3.02	3.30	5.88	5.97	5.91
	Summary Statistics	Mean:	0.97			2.65			3.08			5.91		
		SD:	0.04			0.24			0.16			0.18		
		CV (%):	4.12			9.06			5.19			3.05		
		n	9			9			9			9		

APPENDIX F

Comparison of Methods at LabCorp and USC

All FISH testing was performed using the Vysis PathVysion™ HER-2 DNA Probe Kit. The majority of methods are equivalent for the two laboratories, with only three important procedural differences. The two most significant differences were required because of differences in storage conditions of the archival slides used in these studies (see Table 1).

LabCorp used unused/unstained “back-up” tissue sections remaining in the clinical trial archives. These slides were not coverslipped and were stored at room temperature for possible further use at the time of the original clinical trials. Storage under these conditions for several years rendered the specimens unsuitable for future IHC evaluations. However, because of the inherent stability of target DNA, these slides were suitable for evaluation by FISH. (Note: the storage conditions were suboptimal even for FISH; optimal or routine conditions require that fresh sections be made from the paraffin-embedded tissue block and be mounted on the slide at or near the time of assay, not several years earlier.)

USC used IgG control slides (from either the CTA or estrogen/progesterone receptor assays) that were coverslipped at the time of the original Herceptin clinical trial. The immunostain does not interfere with the analytical specificity of the FISH assay, and the coverslip may have helped to preserve the subcellular architecture of the specimen during the several years of storage. At USC, a substantial amount of soaking in xylene was required to remove the Cytooseal™ cement medium used to mount the coverslip, followed by rinsing in ethanol to remove the excess xylene. If hybridization and interpretation were not successful, tissue sections were subjected to individualized protease digestion under microscopy. This procedure would be expected to optimize hybridization conditions and to potentially provide more consistent signal enumeration.

APPENDIX F (cont'd)
Comparison of Methods at LabCorp and USC

The only difference between the two laboratories that was not related to the differences in storage conditions was the size of the coverslip applied for the probe application. Both laboratories used larger than specified coverslips when required. When a larger coverslip was used, an increase in probe volume was inconsistently employed at LabCorp (due to technician error), but consistently employed at USC. Validation experiments in both laboratories suggested that coverslip size and probe volume across the ranges used in these studies would not be expected to influence FISH scores (see Appendix D). Both laboratories conducted successful batch assays with successful negative and positive controls for both probes.

Table 1
PathVysion™ HER-2 Assay Comparison (LabCorp vs. USC)

Step	Key Parameter	LabCorp	USC	Equivalent	
1	Specimen	Formalin-fixed, paraffin-embedded human breast tissue	Formalin-fixed, paraffin-embedded human breast tissue	Yes	Steps 1–6a represent sample preparation prior to the Vysis PathVysion™ HER-2 Assay
2	Specimen Slide Preparation	4- to 6-µm sections, not coverslipped	Immunostained 4- to 6-µm sections with Cytoseal™ mounting medium and coverslip	No	
3	Specimen Storage	>2.5 years, ambient	>2.5 years, ambient	Yes	
4	Storage & Handling of Reagents	Followed Manufacturer's recommendations	Followed Manufacturer's recommendations	Yes	
5	Working Reagent Preparation	Followed Manufacturer's recommendations	Followed Manufacturer's recommendations	Yes	
6 (6a)	Slide Pretreatment	N/A	Xylene 24–48 hr to remove coverslip, add fresh xylene 48 hr, 100% EtOH to remove xylene	No	
(6b)	Deparaffinization	Hemo-De 3 × 10 min	Hemo-De 3 × 10 min	Yes	Steps 6b–6f: Vysis Paraffin Pretreatment Kit
(6c)	Acid Treatment	0.2N HCl, 20 min	0.2N HCl, 20 min	Yes	
(6d)	Chaotrope Treatment	1M NaSCN 80°C, 60 min (pretreatment soln)	1M NaSCN 80°C, 30 min (pretreatment soln)	Yes	
(6e)	Protease Treatment	Pepsin solution, 37°C, 30 min	Pepsin solution, 37°C, 10–60 min	Yes	
(6f)	Processing	VP 2000 (80%), manual (20%)	VP 2000 (100%)	Yes	

APPENDIX F (cont'd)
Comparison of Methods at LabCorp and USC

Table 1 (cont'd)
PathVysion™ HER-2 Assay Comparison (LabCorp vs. USC)

Step	Key Parameter	LabCorp	USC	Equivalent	
7	Selecting Hybridization Area	Followed Manufacturer's recommendations	Followed Manufacturer's recommendations	Yes	Steps 7–19: Vysis PathVysion™ HER-2 DNA Probe Kit
8	Slide Denaturation	70% Formamide / 2× SSC 72°C, 5 min	70% Formamide / 2× SSC 72 ± 1°C, 5 min	Yes	
9	Probe Application	10–20 µL probe, 22 × 22 mm and 24 × 40 mm coverslips	10–35 µL probe, 22 × 22 mm, 22 × 30, and 24 × 40 mm coverslips	No	
10	Hybridization	37°C humidified, 14–18 hr	37°C humidified, 14–18 hr	Yes	
11	Post-Hybridization Wash	2× SSC/0.3% NP-40 72°C, 2 min	2× SSC/0.3% NP-40 72 ± 1°C, 2 min	Yes	
12	Counterstain	10 µL DAPI, 24×40 mm coverslip	10 µL DAPI, 22×22 mm coverslip	Yes	
13	Slide Storage	–20°C, dark	–20°C, dark	Yes	
14	Assessing Hybridization Quality	Followed Manufacturer's recommendations	Followed Manufacturer's recommendations	Yes	
15	Slide Evaluation				
(15a)	Filters	DAPI/Or/Gr v.2, DAPI/Or v.2, Green single	DAPI/Or/Gr v.2, DAPI/Or v.2, DAPI/Gr	Yes	
(15b)	Microscope objectives	10×, 40×, 100× oil	5×, 10×, 20×, oil: 40×, 63×, 100×	Yes	
(15c)	Mercury bulb	100 watt, hr not specified	100 watt, <200 hr	Yes	
(15d)	Signal Enumeration Guidelines	Not specified	Manufacturer's guidelines	Yes	
16	Nuclei scored	40 (60 if ratio = 1.8–2.2)	60	Yes	
17	Ratio calculation	Total HER2/total CEP 17	Average HER2/average CEP 17	Yes	
18	Establishing HER2 Amplification	Ratio <2.0 non-amplified Ratio ≥2.0 amplified	Ratio <2.0 non-amplified Ratio ≥2.0 amplified	Yes	
19	Control Slides				
(19a)	Specimen	Formalin-fixed, paraffin-embedded breast cancer tissue previously identified as (HER2) positive by IHC or FISH	Internal control (benign and normal cells) and non-amplified and amplified cell lines. Also, ProbeChek HER2	Yes	
(19b)	Performance requirements	Not specified	At least 2 controls w/ successful assay results		

Shading indicates important difference in assay parameter.

APPENDIX G

Clinical Outcomes Based on the Secondary Analysis

For the reporting of results, Genentech defined the “primary” dataset as the original 540 results from LabCorp combined with the 225 additional results from USC (total n = 765/799 results). The results are shown in Section 4.7. However, after completion of the validation process, Genentech defined a “secondary” dataset that gave precedence to results from USC. For any patient with a FISH result from both laboratories, the USC result was used for the secondary dataset. If no result was available from USC, the LabCorp result was used. The tables and Kaplan-Meier plots presented in this appendix are based on this secondary dataset.

Study H0649g: Efficacy Outcomes for the FISH+ and FISH– Subpopulations Defined by the Secondary FISH Analysis

Overall, 163 patients were classified as FISH+ by the primary FISH analysis. One hundred seventy-three patients were classified as FISH+ by the secondary FISH analysis. The efficacy outcomes for the FISH+ subpopulation are summarized in Table 1 (objective response rate) and Figures 1 and 2 (time to disease progression and survival time, respectively). Comparison of the efficacy outcomes based on the secondary FISH analysis with those based on the primary analysis (see Section 4.7.1) indicates that the point estimates and confidence intervals for response rate, median time to disease progression, and median survival time were not affected by the change in definition of FISH status.

Overall, 46 patients were classified as FISH– by the primary FISH analysis and 36 patients were classified as FISH– by the secondary FISH analysis. The efficacy outcomes for the FISH– subpopulation are summarized in Table 1 (objective response rate) and Figures 1 and 2 (time to disease progression and survival time, respectively). The point estimates and confidence intervals for response rate, median time to disease progression, and median survival time were not affected by the change in definition of FISH status.

APPENDIX G (cont'd)
Clinical Outcomes Based on the Secondary Analysis

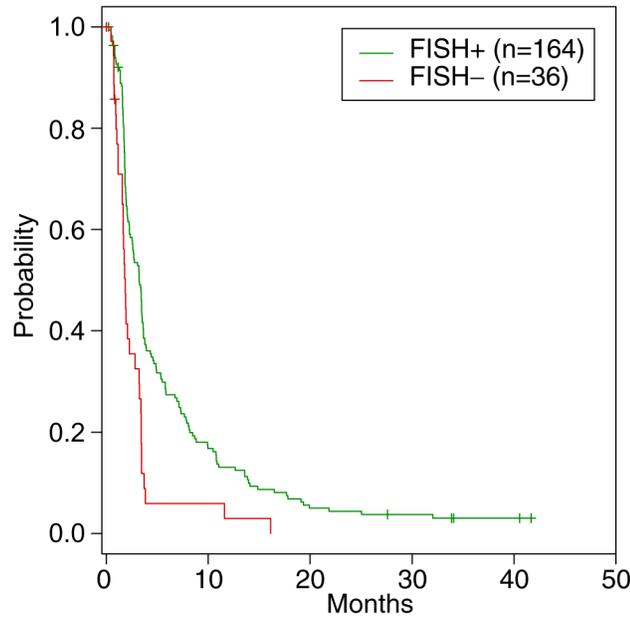
Table 1

Overall Response Rate in Study H0649g for
the Newly Defined Patient Subpopulations:
Secondary Analysis

FISH+	
n/N	33/173
%	19
95% CI	(13.5, 25.7)
FISH-	
n/N	0/36
%	0
95% CI	(0.0, 9.7)

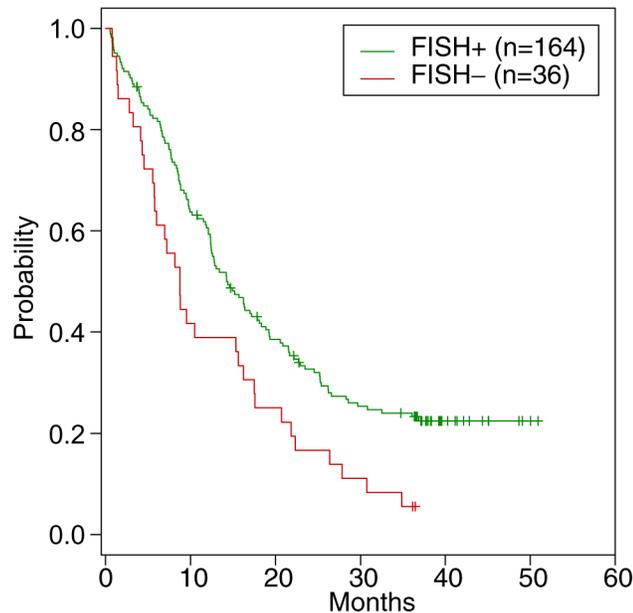
Figure 1

Time to Disease Progression for FISH+ and FISH- Patients in Study H0649g:
Secondary Analysis



APPENDIX G (cont'd)
Clinical Outcomes Based on the Secondary Analysis

Figure 2
 Survival Time for FISH+ and FISH- Patients in Study H0649g:
 Secondary Analysis



Study H0648g: Efficacy Outcomes for the FISH+ and FISH- Subpopulations Defined by the Secondary FISH Analysis

Overall, 325 patients were classified as FISH+ by the primary FISH analysis (164 patients in the Herceptin + chemotherapy treatment group and 161 patients in the chemotherapy alone treatment group). Three hundred forty-five patients were classified as FISH+ based on the secondary analysis (176 patients from the Herceptin + chemotherapy treatment group and 169 patients from the chemotherapy alone treatment group). Objective response rate for the FISH+ subpopulation is summarized in Table 2; Figures 3 and 5 show time to disease progression and survival time for the FISH+ subpopulation, respectively. Comparison of the efficacy outcomes based on the secondary FISH analysis with those based on the primary analysis (see Section 4.7.2) indicates that the point estimates and confidence intervals for response rate, median time to disease

APPENDIX G (cont'd)
Clinical Outcomes Based on the Secondary Analysis

progression, and median survival time were not affected by the change in definition of FISH status.

Overall, 126 patients were classified as FISH– by the primary FISH analysis (62 patients from the Herceptin + chemotherapy treatment group and 64 patients from the chemotherapy alone treatment group). By the secondary FISH analysis, only 106 patients were classified as FISH– (50 patients from the Herceptin + chemotherapy treatment group and 56 patients from the chemotherapy alone treatment group). Objective response rate for the FISH– subpopulation is summarized in Table 2; Figures 4 and 6 show time to disease progression and survival time for the FISH– subpopulation, respectively. The point estimates and confidence intervals for response rate, median time to disease progression, and median survival time were not affected by the change in definition of FISH status.

Table 2
 Overall Response Rate in Study H0648g for the Newly Defined Patient
 Subpopulations: Secondary Analysis

	Herceptin +AC	AC Alone	Herceptin +Paclitaxel	Paclitaxel Alone	Herceptin +Chemo	Chemo Alone
FISH+						
n/N	61/107	39/93	34/69	13/76	95/176	52/169
%	57	42	49	71	54	31
95% CI	(47.6, 66.4)	(31.9, 52.0)	(37.5, 61.1)	(8.6, 25.6)	(46.6, 61.3)	(23.8, 37.7)
p-value (χ^2)	0.0335		<0.0001		<0.0001	
FISH–						
n/N	15/31	19/39	4/19	2/17	19/50	21/56
%	48	49	21	12	38	38
95% CI	(30.8, 66.0)	(33.0, 64.4)	(2.7, 39.4)	(0.0, 27.1)	(24.5, 51.5)	(24.8, 50.2)
p-value (χ^2)	0.9781		0.4554		0.9577	

APPENDIX G (cont'd)

Clinical Outcomes Based on the Secondary Analysis

Figure 3

Time to Disease Progression for FISH+ Patients in Study H0648g: Herceptin + Chemotherapy vs. Chemotherapy Alone: Secondary Analysis

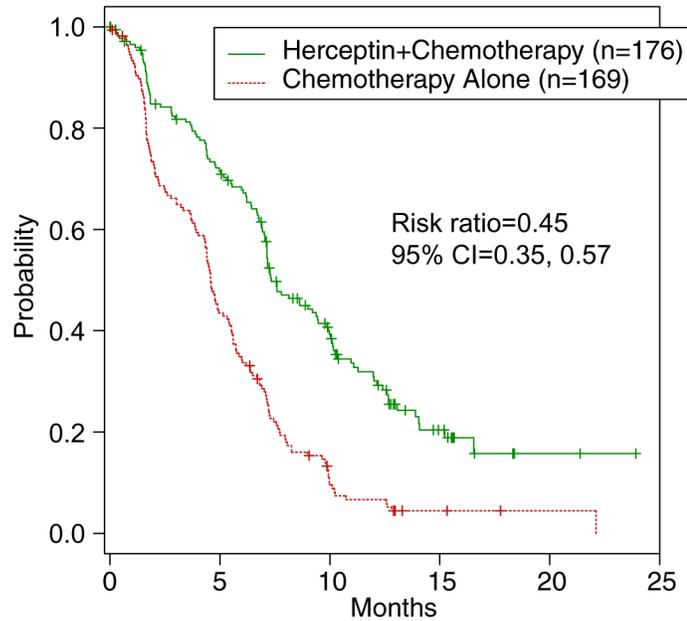
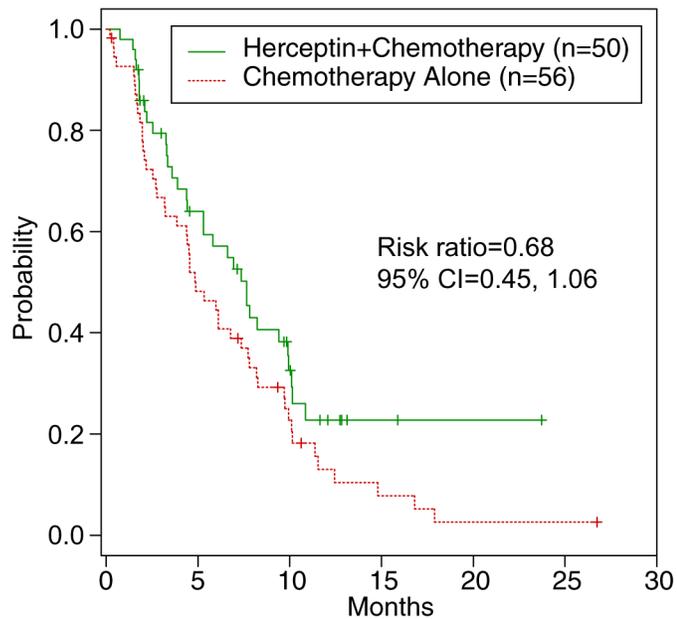


Figure 4

Time to Disease Progression for FISH- Patients in Study H0648g: Herceptin + Chemotherapy vs. Chemotherapy Alone: Secondary Analysis



APPENDIX G (cont'd)
Clinical Outcomes Based on the Secondary Analysis

Figure 5

Survival Time for FISH+ Patients in Study H0648g: Herceptin + Chemotherapy vs. Chemotherapy Alone: Secondary Analysis

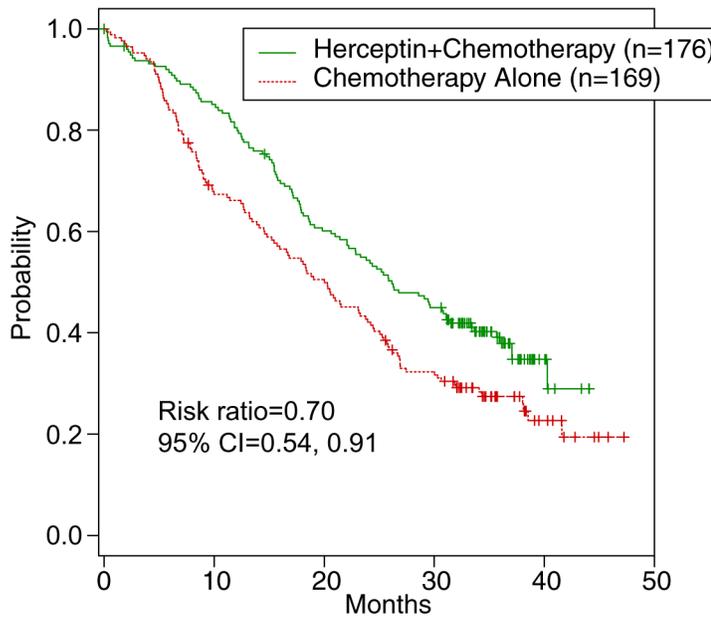


Figure 6

Survival Time for FISH- Patients in Study H0648g: Herceptin + Chemotherapy vs. Chemotherapy Alone: Secondary Analysis

