

**PathVysion™ HER-2 DNA Probe Kit**  
**(LSI® HER-2/*neu* SpectrumOrange™ / CEP® 17 SpectrumGreen™)**  
**Order Number 30-161060 (20 assay)/35-161060 (50 assay)/36-161060 (100 assay)**

**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, 50 or 100 assays dependent on product ordered. 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.: 30-171060/35-171060  
Quantity: 200 µL/500 µL/ 500 µL x 2 for the 100 assay kit  
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.: 30-804840/30-804860/30-804960  
Quantity: 300 µL/600 µL/1000 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

- Precleaned silanized or positively charged glass microscope slides
- Slide warmer (45 - 50°C)
- 22 mm x 22 mm glass coverslips
- Microliter 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  $\geq 0.75$  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 affected 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 µL 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 µm 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.

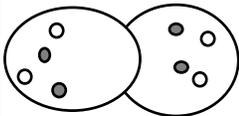
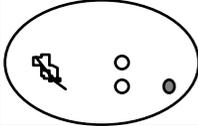
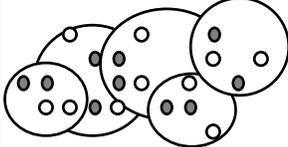
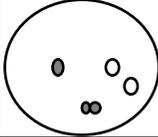
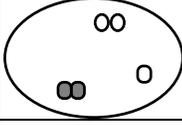
##### Fixing the Specimen

- 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.



**Figure 1**  
**Dual Color Signal Counting Guide**

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

|          |   |  |
|----------|---|--|
| <b>1</b> |    | 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. |
| <b>2</b> |    | Count as two orange signals and two green signals. One orange signal is diffuse.   |
| <b>3</b> |    | Don't count. Nuclei are overlapping, all areas of nuclei are not visible and some signals are in overlapping area.   |
| <b>4</b> |    | Count as two orange signals and two green signals. One orange signal is split.   |
| <b>5</b> |    | Count as one orange signal and two green signals. One green signal is split and the orange signal is split.  |
| <b>6</b> |   | Count as two orange signals and one green signal.  |
| <b>7</b> |  | Count as three orange signals and one green signal.  |
| <b>8</b> |  | 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 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.

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

| Normal Ratio Control Slide                    |                           |           |           |           |           |            |
|---|---------------------------|-----------|-----------|-----------|-----------|------------|
|   | 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 Slide |                           |           |           |           |           |            |
|   | 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                   | 8.0-11.0  | 61.0-72.0 | 4.0-6.0   | 16.0-19.0 | 0.0-2.0    |
| CEP 17  | 0.0-1.0                   | 65.0-75.0 | 25.0-29.0 | 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 Cause   | Solution   |
|---|--|--|
| • No signal or weak signals                           | <ul style="list-style-type: none"> <li>• Inappropriate filter set used to view slides</li> <li>• Microscope not functioning properly</li> <li>• Improper lamps (i.e. Xenon or Tungsten)</li> <li>• Mercury lamp too old</li> <li>• Mercury lamp misaligned</li> <li>• Dirty and/or cracked collector lenses</li> <li>• Dirty or broken mirror in lamp house</li> <li>• Hybridization conditions inappropriate</li> <li>• Inappropriate post-hybridization wash temperature</li> <li>• Air bubbles trapped under coverslip and prevented probe access</li> <li>• Insufficient amount of hybridization solution for section</li> <li>• Inadequate protease digestion</li> <li>• Section overfixed (cell boundaries will be distinct)</li> <li>• DNA loss (poor DAPI staining)</li> </ul> | <ul style="list-style-type: none"> <li>• Use recommended filters</li> <li>• Call microscope manufacturer's technical representative</li> <li>• Use a mercury lamp (100 watt recommended)</li> <li>• Replace with a new lamp</li> <li>• Realign lamp</li> <li>• Clean and replace lens</li> <li>• Clean or replace mirror</li> <li>• Check temp of 37±1°C incubator</li> <li>• Increase hybridization time to at least 14 hours</li> <li>• Check temp. of 72±1°C water bath</li> <li>• Apply coverslip by first touching the surface of the hybridization mixture</li> <li>• Increase amount of hybridization solution to 20 µL per section</li> <li>• Check temp. of 37±1°C bath</li> <li>• Check that pH of buffer is 2.0±0.2</li> <li>• Increase digestion time, up to 60 min.</li> <li>• Eliminate fixation</li> <li>• Optimal fixation time in preparation of paraffin embedded slides is 24-48 hrs. Longer fixation times will lead to progressive degradation of signal intensity.</li> <li>• Check fixation conditions</li> </ul> |
| • Variation of signal intensity across tissue section | <ul style="list-style-type: none"> <li>• Inherent in many tissue sections</li> <li>• Probe unevenly distributed on slide due to air bubbles under coverslip</li> <li>• Oversized section</li> </ul>  | <ul style="list-style-type: none"> <li>• Check DAPI staining. If DAPI staining in poor area is good, then score slide. If DAPI staining is poor in poor area, increase fixation time</li> <li>• Repeat hybridization on next adjacent section or same slide and make sure no air bubbles are trapped under coverslip</li> <li>• Increase volume of hybridization solution to 20 µL on large tissue sections</li> </ul>   |
| • Noisy background                                    | <ul style="list-style-type: none"> <li>• Inadequate wash stringency</li> </ul>   | <ul style="list-style-type: none"> <li>• Check pH of 7.2-7.5 wash buffer</li> <li>• Check temperature of 72±1°C bath</li> <li>• Provide gentle agitation during wash</li> <li>• Increase wash time to 5 minutes</li> </ul>   |
| • Tissue loss or tissue morphology degraded           | <ul style="list-style-type: none"> <li>• Tissue section underfixed (poor DAPI staining )</li> <li>• Inappropriate slides used</li> <li>• Improper slide baking</li> <li>• Over-pretreatment</li> <li>• Over-denaturation</li> <li>• Tissue section was torn removing coverslip after hybridization</li> </ul>  | <ul style="list-style-type: none"> <li>• Check fixation time/conditions</li> <li>• Use positively charged slides</li> <li>• Check temp. of 56°C oven</li> <li>• Check temp. of 80±1°C pretreatment</li> <li>• Decrease pretreatment time</li> <li>• Decrease protease digestion time</li> <li>• Check temp. of 72±1°C denaturation bath</li> <li>• Decrease denaturation time</li> <li>• Allow coverslip to soak off in wash buffer</li> </ul>   |

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.

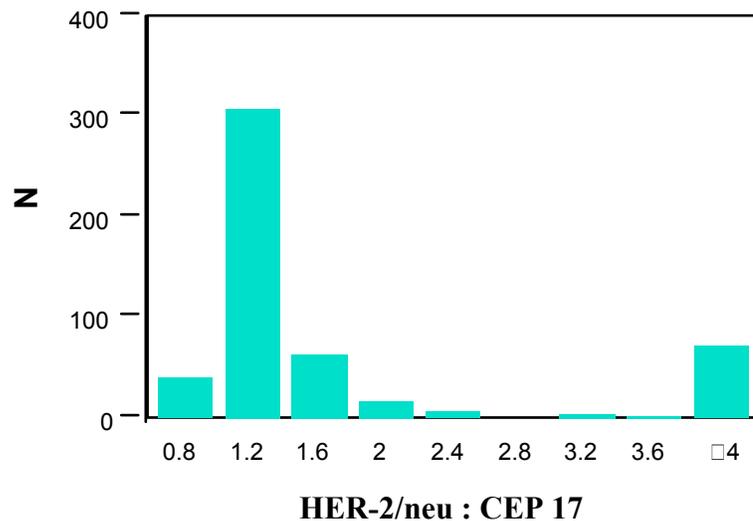
**Table 4**  
**Distribution of Ratio of HER-2/*neu* to CEP 17 Signals in Amplified Breast Tissue Specimens**

| 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 ProbeChek™ 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 ( $\pm 0.03$ ), and the 1.6 - 2.0 HER-2/*neu*:CEP 17 ratio specimen was estimated with a mean of 1.81 ( $\pm 0.08$ ). 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          | 8       | 8       | 8       |
| 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          | 8       | 8       | 8       |
| 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          | 8       | 8       | 8       |
| 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          | 8       | 8       | 8       |

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       | 7.67   | 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/ <i>neu</i> 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         | 4.48         | 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        | 11.22        | 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/ <i>neu</i> 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/*neu* gene amplification. The specimens included one normal (no amplification), two with low level, and one with moderate level HER-2/*neu* 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/*neu* 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/*neu* 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       | <b>1.01</b>  | <b>1.05</b>  | <b>1.04</b>  | 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       | <b>2.53</b>  | <b>2.42</b>  | <b>2.42</b>  | 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       | <b>3.17</b>  | <b>2.98</b>  | <b>3.03</b>  | 0.5815  |
|                | 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       | <b>5.66</b>  | <b>5.60</b>  | <b>5.62</b>  | 0.9652  |
|                | 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/*neu* 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        | <b>Mean</b> | <b>1.00</b> | <b>1.15</b> | <b>1.01</b> | <b>1.04</b> | <b>0.98</b> | 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        | <b>Mean</b> | <b>2.39</b> | <b>2.45</b> | <b>2.55</b> | <b>2.26</b> | <b>2.65</b> | 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        | <b>Mean</b> | <b>3.00</b> | <b>3.09</b> | <b>3.41</b> | <b>2.73</b> | <b>3.08</b> | 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        | <b>Mean</b> | <b>5.42</b> | <b>5.19</b> | <b>5.89</b> | <b>5.73</b> | <b>5.91</b> | <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/ <i>neu</i> 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               | 10.98    | 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/m<sup>2</sup>, doxorubicin at 60 mg/m<sup>2</sup>, and 5-fluorouracil at 600 mg/m<sup>2</sup> for four cycles), a moderate dose (cyclophosphamide at 400 mg/m<sup>2</sup>, doxorubicin at 40 mg/m<sup>2</sup>, and 5-fluorouracil at 400 mg/m<sup>2</sup> for six cycles), or a low dose (cyclophosphamide at 300 mg/m<sup>2</sup>, doxorubicin at 30 mg/m<sup>2</sup>, and 5-fluorouracil at 300 mg/m<sup>2</sup> 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/ <i>neu</i> amplification*<br>n=91 | No HER-2/ <i>neu</i> amplification*<br>n=433 |
|---------------------------|--|--|
| Age                       |  |  |
| <40                       | 17.6                                     | 14.5   |
| 40-50                     | 39.6                                     | 40.0   |
| >50                       | 42.9                                     | 48.5   |
| Premenopausal             | 46.2                                     | 39.5   |
| 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            |  |  |
| ≤3                        | 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                  | 34.1                                     | 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                | 12.93 | 0.0003  |
| Premenopausal                       | 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                | 7.15  | 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%, 75%, 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**

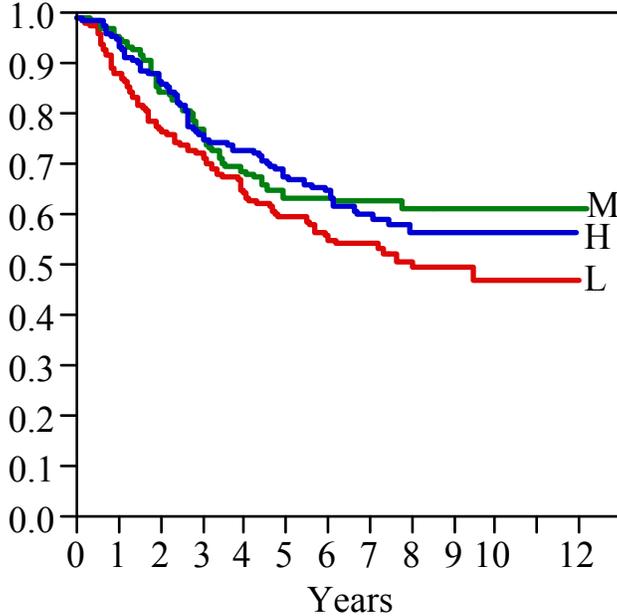
| CAF Dose | HER-2/ <i>neu</i> negative | HER-2/ <i>neu</i> positive |
|----------|----------------------------|----------------------------|
| Low      | 55%                        | 36%                        |
| Moderate | 63%                        | 44%                        |
| High     | 61%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

**Table 15**  
**Overall Survival Probabilities**

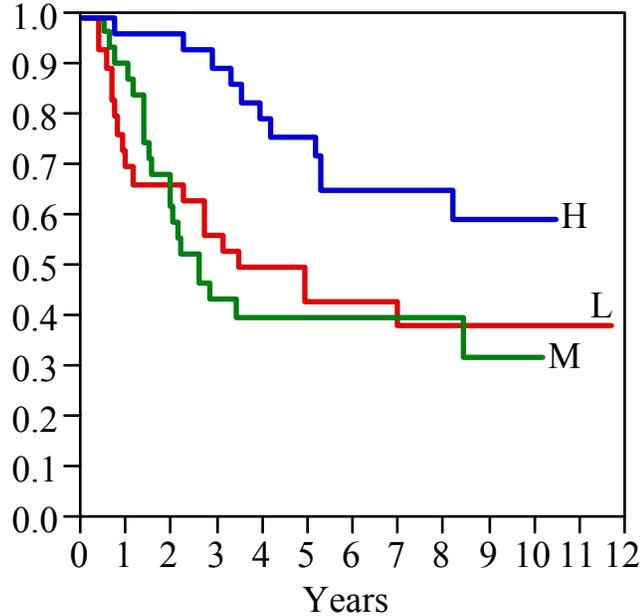
| CAF Dose | HER-2/ <i>neu</i> negative | HER-2/ <i>neu</i> positive |
|----------|----------------------------|----------------------------|
| Low      | 64%                        | 48%                        |
| Moderate | 75%                        | 50%                        |
| High     | 70%                        | 76%                        |

**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\***

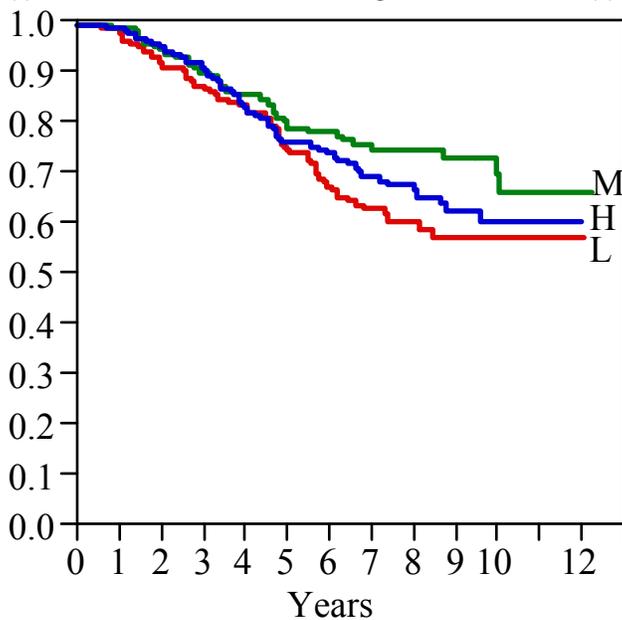
(a) Disease-free survival for HER-2/*neu*-negative



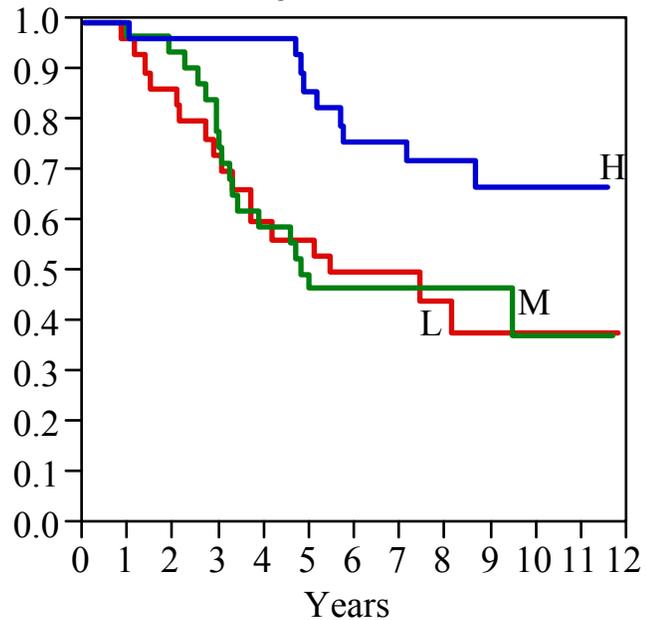
(b) Disease-free survival for HER-2/*neu*-positive



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



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



\*HER-2/neu positivity means HER/CEP  $\geq 2$ . 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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