PathVysion HER-2 DNA Probe Kit

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REF 02J01

30-608377/R7

PathVysion

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Key to Symbols Used

	Manufacturer
REF	Reference Number
IVD	In Vitro Diagnostic Medical Device
Σ	Contains sufficient for <n> tests
-20°C / -20°C	Temperature limitation
25°C / -20°C	Temperature limitation
	Biological Risks
	Danger
	Danger
	Danger
	Consult instructions for use
	Use by
EC REP	Authorized representative in the European Community

PATHVYSION HER-2 DNA PROBE KIT

Part No. 30-161060, List No. 02J01-030 (20 assays)/
Part No. 35-161060, List No. 02J01-035 (50 assays)/
Part No. 36-161060, List No. 02J01-036 (100 assays)

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.

The PathVysion Kit is indicated as an aid in the assessment of patients for whom HERCEPTIN® (Trastuzumab) treatment is being considered (see HERCEPTIN package insert).

Warning:

HERCEPTIN therapy selection

NOTE: All of the patients in the HERCEPTIN clinical trials were selected using an investigational immunohistochemical assay (CTA). None of the patients in those trials were selected using the PathVysion assay. The PathVysion assay was compared to the CTA on a subset of clinical trial samples and found to provide acceptably concordant results. The actual correlation of the PathVysion assay to HERCEPTIN clinical outcome in prospective clinical trials has not been established.

Adjuvant therapy selection

The 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 and no treatment decision for stage II, node-positive breast cancer patients should be based on HER-2/*neu* gene amplification status alone.

Selected patients with breast cancers shown to lack amplification of HER-2/*neu* may still benefit from CAF (cyclophosphamide, doxorubicin, 5-fluorouracil) adjuvant therapy on the basis of other prognostic factors that predict poor outcome (eg, tumor size, number of involved lymph nodes, and hormone receptor status). Conversely, selected patients with breast cancers shown to contain gene amplification may not be candidates for CAF therapy due to pre-existing or intercurrent medical illnesses.

Required Training

Abbott Molecular will provide training in specimen preparation, assay procedure, and interpretation of FISH testing of the HER-2 gene for inexperienced users. It is also recommended that a laboratory that has previously received training but now has new personnel performing the assay request training for the new users.

SUMMARY AND EXPLANATION

Among all cancers in the United States there were approximately 2,591,855 women alive who had a history of cancer of the breast in 2007. This includes any person alive who had been diagnosed with cancer of the breast at any point prior to 2007 and includes persons with active disease and those who are cured of their disease.[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.

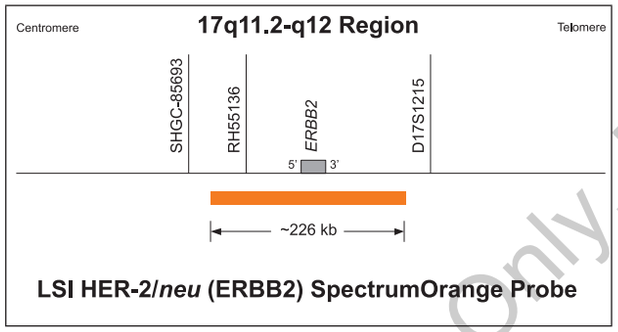
The FISH technique has been used to detect HER-2/*neu* gene amplification in human breast carcinoma cell lines in both interphase and metaphase cells.[11-14] FISH appears to be an alternative technique capable of overcoming many of the inherent technical and interpretative limitations of other techniques, 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.

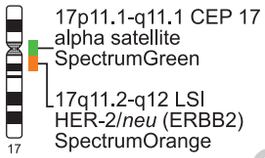
PRINCIPLES OF THE PROCEDURE

In situ hybridization is a technique that allows the visualization of specific nucleic acid sequences within a cellular preparation. Specifically, DNA FISH involves the precise annealing of a single-stranded, fluorescently-labeled DNA probe to complementary target sequences. The hybridization of the probe with the cellular DNA site is visible by direct detection using fluorescence microscopy.

The Locus Specific Identifier (LSI) HER-2/*neu* DNA probe is a 226 Kb SpectrumOrange directly-labeled, fluorescent DNA probe specific for the HER-2/*neu* gene locus (17q11.2-q12). The Chromosome Enumeration Probe (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. Unlabeled blocking DNA is also included with the probes to suppress sequences contained within the target loci that are common to other chromosomes. This PathVysion Kit is designed for the detection of HER-2/*neu* gene amplification in formalin-fixed, paraffin-embedded human breast tissue specimens by FISH. 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.

Formalin-fixed, paraffin-embedded tissue specimens are placed on slides. The DNA is denatured to single-stranded form and subsequently allowed to hybridize with the PathVysion probes. Following hybridization, the unbound probe is removed by a series of washes and the nuclei are counterstained with DAPI (4,6 diamidino-2-phenylindole), a DNA-specific stain that fluoresces blue. Hybridization of the PathVysion probes is viewed using a fluorescence microscope equipped with appropriate excitation and emission filters allowing visualization of the intense orange and green fluorescent signals. Enumeration of the LSI HER-2/*neu* and CEP 17 signals is conducted by microscopic examination of the nucleus, which yields a ratio of the HER-2/*neu* gene to chromosome 17 copy number.





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 × 22 mm target area.

1. LSI HER-2/neu SpectrumOrange (low copy number E. coli vector)/ CEP 17 SpectrumGreen DNA Probe (E. coli plasmid)

Part Number: 30-171060/35-171060

Quantity: 200 µL/500 µL/500 µL × 2 for the 100 assay kit

Storage: -20°C / -20°C – 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

Part Number: 30-804840/30-804860/30-804960

Quantity: 300 µL/600 µL/1000 µL

Storage: -20°C / -20°C – 20°C in the dark

Composition: 1000 ng/mL DAPI (4,6-diamidino-2-phenylindole) in phenylenediamine dihydrochloride, glycerol, and buffer.

3. NP-40

Part Number: 30-804820

Quantity: 4 mL (2 vials)

Storage: -20°C / 25°C – 20 to 25°C

Composition: Igepal (NP-40 substitute) [Octyl phenoxy] polyethoxyethanol.

4. 20X SSC salts

Part Number 30-805850

Quantity: 66 g for up to 250 mL of 20X SSC solution

Storage: -20°C / 25°C – 20 to 25°C

Composition: Sodium chloride and sodium citrate.

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 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) Part No. 30-805093, List No. 02J05-030 (manual assay) or Part No. 32-805093, List No. 02J05-010 (for use with Vysis AutoVysion System) Formalin-fixed, paraffin-embedded, cultured human breast cancer cell line (MDA-MB-231; normal LSI HER-2/*neu*:CEP 17 ratio) applied to glass microscope slides. Quantity: 5 slides. Store the control slides at 15 to 30°C in a sealed container with desiccant to protect them from humidity.
- ProbeChek HER-2/*neu* Cutoff Control Slides (Weakly Amplified Signal Ratio) Part No. 30-805042, List No. 02J04-030 (manual assay) or Part No. 32-805042, List No. 02J04-010 (for use with Vysis AutoVysion System) Formalin-fixed, paraffin-embedded, cultured human breast cancer cell line (Hs 578T; low level HER-2/*neu* amplification) applied to glass microscope slides. Quantity: 5 slides. Store the control slides at 15 to 30°C in a sealed container with desiccant to protect them from humidity.
- Vysis Paraffin Pretreatment Reagent Kit (Part No. 32-801200, List No. 02J02-032) which includes:
  - Vysis Pretreatment Solution (NaSCN) Quantity: 5 × 50 mL
  - Vysis Protease (Pepsin (Activity 1:3000 to 1:3500) Quantity: 5 × 25 mg

  NOTE: Pepsin digests not less than 3000 and not more than 3500 times its weight of coagulated egg albumin.
  - Vysis Protease Buffer (NaCl solution, pH 2.0) Quantity: 5 × 50 mL
  - Vysis Wash Buffer (2X SSC, pH 7.0) Quantity: 2 × 250 mL
- Neutral buffered formalin solution (4% formaldehyde in PBS)
- Hemo-De clearing agent (Scientific Safety Solvents #HD-150)
- Hematoxalin and eosin (H & E)
- Immersion oil appropriate for fluorescence microscopy. Store at room temperature (15 to 30°C).
- Ultra-pure, formamide.
- 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

NOTE: Where storage conditions are not listed, store reagents per vendor recommendation.

Laboratory Equipment

- Precleaned silanized or positively charged glass microscope slides
- Slide warmer (45 to 50˚C)
- 22 mm × 22 mm glass coverslips
- Microliter pipettor (1 to 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 ± 2˚C)
- Air incubators (37 ± 1˚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
- Fluorescence microscope equipped with recommended filters (see next section)
- pH meter
- 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 FISH 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 25X objective, in conjunction with 10X eyepieces, is suitable for locating target. 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 Abbott for most microscope models. The recommended filter sets for the PathVysion Kit are the DAPI/9-Orange dual-bandpass, DAPI/Green dual-bandpass, Green/Orange (V.2) dual-bandpass, and the DAPI/Green/Orange (V.2) 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 7.0 to 8.0. This solution can be used for up to 1 week. Check pH prior to each use. Store at 2 to 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 1 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 to 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

IVD In Vitro Diagnostic Medical Device

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 ProbeChek control slides 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 Abbott Molecular 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. 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.
12. LSI HER-2/*neu* and CEP 17 DNA probe mixture contains formamide, a teratogen. Avoid contact with skin and mucous membranes.
13. Calibrated thermometers are required for measuring temperatures of solutions, water baths, and incubators.
14. 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.
15. All hazardous materials should be disposed of according to your institution's guidelines for hazardous disposal.

PathVysion LSI HER-2/neu SpectrumOrange/ CEP 17 SpectrumGreen Probes

CAUTION: This preparation contains human sourced and/or potentially infectious components. No known test method can offer complete assurance that products derived from human sources or inactivated microorganisms will not transmit infection. These reagents and human specimens should be handled as if infectious using safe laboratory procedures, such as those outlined in Biosafety in Microbiological and Biomedical Laboratories,[21] OSHA Standards on Bloodborne Pathogens,[22] CLSI Document M29-A3,[23] and other appropriate biosafety practices.[24] Therefore all human sourced materials should be considered infectious.

These precautions include, but are not limited to, the following:

- Wear gloves when handling specimens or reagents.
- Do not pipette by mouth.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in areas where these materials are handled.
- Clean and disinfect spills of specimens by including the use of a tuberculocidal disinfectant such as 1.0% sodium hypochlorite or other suitable disinfectant.[21]
- Decontaminate and dispose of all potentially infectious materials in accordance with local, state, and federal regulations.[24]

PathVysion LSI HER-2/neu SpectrumOrange/ CEP 17 SpectrumGreen Probes

Danger

Hazard-determining components of labeling: Formamide

H360D	May damage the unborn child.
P201	Obtain special instructions before use.
P281	Use personal protective equipment as required.
P303+ P361+ P353	IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.
P308+P313	IF exposed or concerned: Get medical advice/attention.
P403+P235	Store in a well-ventilated place. Keep cool.
P501	This material and its container must be disposed of in a safe way.

NP-40

Danger

Hazard-determining components of labeling: Triton X-100

H302	Harmful if swallowed.
H318	Causes serious eye damage.
H412	Harmful to aquatic life with long lasting effects.
P280	Wear protective gloves/protective clothing/ eye protection.
P264	Wash hands thoroughly after handling.
P273	Avoid release to the environment.
P305+ P351+ P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P301+P312	IF SWALLOWED: Call a poison center or doctor/physician if you feel unwell.
P501	This material and its container must be disposed of in a safe way.

Safety Data Sheet Statement: Important information regarding the safe handling, transport, and disposal of this product is contained in the Safety Data Sheet.

NOTE: Material Safety Data Sheets (MSDS) for all reagents provided in the kits are available upon request from the Abbott Molecular Technical Services Department.

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 area for the PathVysion assay should be performed by a 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 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, 50, or 100 assays; 1 assay for the PathVysion Kit is defined as a 22 mm × 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 to 6 µm thick paraffin sections using a microtome.
2. Float the sections in a protein-free water bath at 40 ± 2°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 (Part No. 32-801200, List No. 02J02-032) 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% ethanol for 5 minutes at room temperature. Repeat.
- Air-dry slides or place slides on a 45 to 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 ± 1°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 ± 1°C for 10 to 60 minutes.
- Immerse slides in Wash Buffer for 5 minutes. Repeat.
- Dry slides on a 45 to 50°C slide warmer for 2 to 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 to 50°C slide warmer for 2 to 5 minutes.
- Proceed with the PathVysion assay protocol.

ASSAY PROCEDURE: FISH PROCEDURE SUMMARY

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 to 3 seconds in a bench-top microcentrifuge to bring the contents to the bottom of the tube. Gently vortex again to mix.

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 × 3 in taped to the side of the container) to 37 ± 1°C by placing it in the 37 ± 1°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 to 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 1 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 to 50°C slide warmer for 2 to 5 minutes.

Hybridization

1. Apply 10 µL of probe mixture to target area of slide. Immediately place a 22 mm × 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, forming a seal around the coverslip.
3. Place slides in the prewarmed humidified hybridization chamber. Cover the chamber with a tight lid and incubate at 37 ± 1°C overnight (14 to 18 hours).
4. Proceed to Post-Hybridization Washes.

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 signals 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 filters. 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

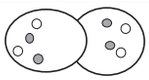
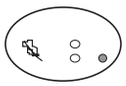
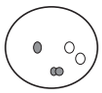
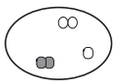
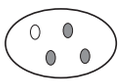
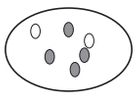
Using a 40X objective, scan several areas of tumor cells to account for possible heterogeneity. Select an area of good nuclei distribution; avoid areas of the target where hybridization signals are weak. Using a 63X or 100X objective, begin analysis in the upper left quadrant of the selected area and, 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 2 signals that are the same size and separated by a distance equal or less than the diameter of the signal as 1 signal.
- Do not score nuclei with no signals or with signals of only 1 color. Score only those nuclei with 1 or more FISH signals of each color.
- For each nucleus, count the number of LSI HER-2/*neu* signals and the number of CEP 17 signals.

  NOTE: It may be necessary to alternate between the DAPI/9-orange, DAPI/green, Green/Orange (V.2), and the DAPI/green/Orange (V.2) filter sets to view both color signals. Record counts (see Recommended Method for HER-2 to CEP 17 Ratio Determination).

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 2 orange and 2 green in each nucleus.
2		Count as 2 orange signals and 2 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 2 orange signals and 2 green signals. One orange signal is split.
5		Count as 1 orange signal and 2 green signals. One green signal is split and the orange signal is split.
6		Count as 2 orange signals and 1 green signal.
7		Count as 3 orange signals and 1 green signal.
8		Count as 4 orange signals and 2 green signals.

Recommended Method for LSI HER-2 to CEP 17 Ratio Determination

The recommended method for LSI HER-2/*neu* to CEP 17 ratio determination is by dividing the total number of LSI HER-2/*neu* signals by the total number of CEP 17 signals in counting the same 20 nuclei.

1. Determine and record the number of LSI HER-2/*neu* and CEP 17 counts in 20 nuclei (see sample data sheet, below).
2. Add all LSI HER-2/*neu* signals. This sum represents the Total LSI HER-2/*neu* signals, eg, 252.
3. Add all CEP 17 signals. This sum represents the Total CEP 17 signals, eg, 44.
4. To calculate the final result, use the following ratio.
   Total LSI HER-2/*neu* signals (step 2)/Total CEP 17 signals (step 3), eg, 252/44 equals a ratio of 5.73, which is positive for HER-2/*neu* amplification.
   If the LSI HER-2/*neu* to CEP 17 ratio is borderline (1.8 to 2.2), count an additional 20 nuclei and recalculate the ratio based on the total of 40 nuclei.
5. Report results as follows:
   If the ratio is < 2, HER-2/*neu* gene amplification was not observed; if the ratio is ≥ 2, HER-2/*neu* gene amplification was observed.

NOTE: A ratio at or near the cutoff (1.8 to 2.2) should be interpreted with caution, as described above.

	HER-2/*neu*	CEP 17
1	5	1
2	6	1
3	10	1
4	11	1
5	14	1
6	17	1
7	6	2
8	11	2
9	15	2
10	14	2
11	16	2
12	20	2
13	8	3
14	10	3
15	12	3
16	14	3
17	18	3
18	19	3
19	11	4
20	15	4
Total	**252**	**44**

QUALITY CONTROL

Use of Control Slides

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 Preparation procedure (see Specimen Processing and Slide Preparation section). Controls should be run on each day of FISH testing and with each new PathVysion kit lot. ProbeChek control slides are required with each run of patient slides processed. In addition, individual users may choose to use their own control material, providing it is characterized and validated in accordance with CLIA high-complexity requirements.

Assess control slide adequacy and perform signal enumeration according to the instructions in the Signal Enumeration section. The criteria for slide adequacy must be satisfied and the LSI HER-2/*neu*:CEP 17 ratio results should be within the established ranges for acceptable test performance. See **Table 1** for acceptable ProbeChek slide results.

In no case should FISH results be reported if assay controls fail. 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. If control slides meet the acceptance criteria but the results are outside of the specified range, the enumeration may not have been performed correctly and an independent, repeat analysis of the same slide may be appropriate. In the event of hybridization failure, with either the specimen or control slide(s), consult the **Troubleshooting Guide** in **Table 2**.

For clinical specimens, when interpretation of the hybridization signal is difficult and there is insufficient specimen sample for reassay, the test is uninformative. If there are insufficient cells for analysis, the test is uninformative.

Patient specimens should be controlled according to standard laboratory procedure requirements. Hybridization quality and enumeration results should be documented on an appropriate form. Hybridization quality and efficiency should be considered when evaluating results.

Table 1. Acceptable HER-2/*neu*: CEP 17 Ratio Ranges for ProbeChek HER-2/*neu* Control Slides

Control	HER-2/*neu*:CEP 17 Acceptable Range
Normal Ratio Control Slide (Part No. 30-805093/List No. 02J05-030)	0.75 to 1.25
Cutoff (Weakly Amplified) Ratio Control Slide (Part No. 30-805042/List No. 02J04-030)	1.6 to 2.0

Table 1a. Acceptable HER-2/*neu*: CEP 17 Ratio Ranges for Abbott AutoVysion System (Automated) Enumeration of ProbeChek HER-2/*neu* Control Slides

Control	HER-2/*neu*:CEP 17 Acceptable Range
Normal Ratio Control Slide (Part No. 32-805093/List No. 02J05-010)	0.7 to 1.7
Cutoff (Weakly Amplified) Ratio Control Slide (Part No. 32-805042/List No.02J04-010)	1.5 to 2.6

Table 2. Troubleshooting Guide

Problem	Probable Cause	Solution
• No signal or weak signals	• Inappropriate filter set used to view slides	• Use recommended filters
	• Microscope not functioning properly	• Call microscope manufacturer's technical representative
	• Improper lamps (ie, Xenon or Tungsten)	• Use a mercury lamp (100-watt recommended)
	• Mercury lamp too old	• Replace with a new lamp
	• Mercury lamp misaligned	• Realign lamp
	• Dirty and/or cracked collector lenses	• Clean or replace lens
	• Dirty or broken mirror in lamp house	• Clean or replace mirror
	• Hybridization conditions inappropriate	• Check temperature of 37 ± 1°C incubator
	• Inappropriate post-hybridization wash temperature	• Increase hybridization time to at least 14 hours • Check temperature of 72 ± 1°C solution
	• Air bubbles trapped under coverslip and prevented probe access	• Apply coverslip by first touching the surface of the hybridization mixture
	• Insufficient amount of hybridization solution for section	• Increase amount of hybridization solution to 20 µL per section
	• Inadequate protease digestion	• Check temperature of 37 ± 1°C solution • Check that pH of buffer is 2.0 ± 0.2 • Increase digestion time, up to 60 minutes
	• Section over-fixed (cell boundaries will be distinct)	• Eliminate fixation in pretreatment • Optimal fixation time in preparation of paraffin embedded slides is 24 to 48 hours. Longer fixation times will lead to progressive degradation of signal intensity.
	• DNA loss (poor DAPI staining)	• Check fixation conditions.

Table 2. Troubleshooting Guide (Continued)

Problem	Probable Cause	Solution
• Variation of signal intensity across tissue section	• Inherent in many tissue sections	• 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.
	• Probe unevenly distributed on slide due to air bubbles under coverslip	• Repeat hybridization on next section or same slide and make sure no air bubbles are trapped under coverslip
	• Oversized section	• Increase volume of hybridization solution to 20 µL on large tissue sections.
• Noisy background	• Inadequate wash stringency	• Check pH of 7.0 to 7.5 post-hybridization wash buffer
		• Check temperature of 72±1°C solution
		• Provide gentle agitation during wash
		• Increase wash time to 5 minutes
• Tissue loss or tissue morphology degraded	• Tissue section under fixed (poor DAPI staining)	• Check fixation time/ conditions
	• Inappropriate slides used	• Use positively charged slides
	• Improper slide baking	• Check temperature of 56°C oven
	• Over-pretreatment	• Check temperature of 80±1°C pretreatment solution
		• Decrease pretreatment time
		• Decrease protease digestion time
	• Over-denaturation	• Check temperature of 72±1°C denaturation solution
		• Decrease denaturation time
	• Tissue section was torn removing coverslip after hybridization	• Allow coverslip to soak off in post-hybridization wash buffer

Contact the Abbott Molecular Technical Services Department for further assistance.

INTERPRETATION OF RESULTS

The number of LSI HER-2/*neu* and CEP 17 signals per nucleus are recorded in columns. Results on enumeration of 20 interphase nuclei from tumor cells per target are reported as the ratio of the total HER-2/*neu* signals to those 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 to 2.2) should be interpreted with caution. In the event of a borderline result (1.8 to 2.2), particularly if there also appears to be variability of the counts from nucleus to nucleus, 20 additional nuclei should be enumerated. 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 1 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 20 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 and for metastatic breast cancer patients being considered for HERCEPTIN therapy. 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 analysis was performed on human breast tissue specimens from 524 breast cancer patients to verify the cutoff point and to assess the expected ratio of LSI HER-2/*neu* to CEP 17. Sixty nuclei were enumerated per specimen. Based on a cutoff point of 2.0 for assessing HER-2/*neu* gene amplification in breast tissue specimens (see "Establishment of Cut-off Point", below, for details on determining cutoff point), 433 of the specimens were negative and 91 positive for HER-2/*neu* gene amplification. The distribution of ratios of HER-2/*neu* to CEP 17 signals for the 433 nonamplified specimens is summarized in **Table 3**.

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

	Range		
Statistics	0.1 to 1.0	1.1 to 1.5	1.6 to 1.99
Mean	0.86	1.15	1.72
SD	0.14	0.13	0.11
n	185	226	22

The distribution of ratios of HER-2/*neu* to CEP 17 signals for the 91 HER-2/*neu* amplified specimens is summarized in **Table 4**.

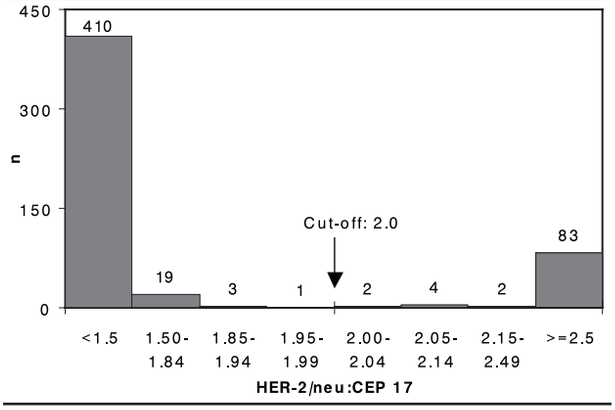
Table 4. Distribution of Ratio of LSI HER-2/*neu* to CEP 17 Signals in Amplified Breast Tissue Specimens

	Range		
Statistics	2.0 to 5.0	5.1 to 10.0	>10.0
Mean	3.35	7.39	12.77
SD	0.95	1.41	1.80
n	33	42	16

Establishment of Cut-off Point

In the pivotal CALGB 8869 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 nonamplified 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 to 1.2 HER-2/*neu*: CEP 17 ratio specimen was estimated with a mean of 1.05 (± 0.03) and the 1.6 to 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 to 1.2 ratio specimen and the lower 95% CI for the 1.6 to 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 from normal lymphocytes according to standard Abbott Molecular 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 2 probes.

Stringency studies were also performed, according to standard Abbott Molecular 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, 3 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 2 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, although there were no statistically significant differences in hybridization quality between 14 and 18 hours. Thus, an incubation time of 14 to 18 hours is recommended. 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. Therefore, the recommended post-hybridization wash conditions are 72 ± 1˚C for 2 minutes. 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 and Repeatability Preclinical Studies

The repeatability of the FISH assay for HER-2/*neu* was determined on consecutive sections of normal and amplified breast tissue, as well as on different thicknesses of the same tissue. On 10 consecutive tissue sections from one normal breast tissue, the average ratio of LSI HER-2/*neu* to CEP 17 copy number was 1.19 (SD = 0.05); the results are shown in **Table 5**.

Table 5. Average Number of Signals per Cell and Ratio of LSI HER-2/*neu*: CEP 17 Copy Number in Consecutive Sections (with normal HER-2/*neu*)

	Section Number									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
LSI HER-2	3.8	3.2	3.6	3.5	3.6	3.5	3.4	3.5	3.3	3.1
CEP 17	3.1	3.1	3.0	2.7	3.0	3.0	2.8	2.1	3.0	2.7
Ratio	1.2	1.2	1.2	1.3	1.2	1.2	1.2	1.6	1.1	1.1

On 10 consecutive tissue sections from 1 specimen with amplified HER-2/*neu*, the average ratio of HER-2/*neu* to CEP 17 copy number was 3.61 (SD = 0.50); the results are shown in **Table 6**.

Table 6. Average Number of Signals per Cell and Ratio of LSI HER-2/*neu*: CEP 17 Copy Number in Consecutive Sections (with amplified HER-2/*neu*)

	Section Number									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
LSI HER-2	4.7	4.9	5.9	4.5	3.6	4.6	4.6	4.8	4.5	4.2
CEP 17	1.2	1.3	1.3	1.3	1.3	1.3	1.4	1.3	1.4	1.3
Ratio	3.9	3.7	4.7	3.6	2.8	3.7	3.3	3.8	3.3	3.3

Similarly, on 8 consecutive normal tissue sections of different thickness (2 to 8 microns), the average ratio of HER-2/*neu* to CEP 17 copy number was 1.15 (SD = 0.16); the results are shown in **Table 7**. These results demonstrated an acceptable degree of reproducibility of the HER-2/*neu* FISH assay in tissue sections with thicknesses between 4 and 8 microns.

Table 7. Average Number of Signals per Cell and Ratio of LSI HER-2/*neu*: CEP 17 Copy Number in Consecutive Sections of Different Thickness

	Thickness of Section (microns)							
	2	**2**	**4**	**4**	**6**	**6**	**8**	**8**
LSI HER-2	2.3	2.4	2.4	2.7	2.7	2.8	2.6	3.3
CEP 17	1.7	1.8	2.3	2.5	2.7	2.7	2.5	3.2
Ratio	1.4	1.4	1.1	1.1	1.0	1.1	1.1	1.0

Reproducibility Using Control Slides

To assess the reproducibility of the LSI HER-2/*neu* and CEP 17 assay, analyses for the ratio of LSI 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 to 1.2) and amplified (1.6 to 2.0, 3 to 5, 7 to 11) ratios of LSI HER-2/*neu* to CEP 17 were evaluated for LSI 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 2 replacement slides was successful.

Using ANOVA, statistically significant variations were observed between observers, which reflect 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 LSI HER-2/*neu* to CEP 17 are shown in **Tables 8** through **11**.

Table 8. Site-to-Site Reproducibility

Ratio of LSI HER-2/*neu* to CEP 17	Statistics	Site No. 1	Site No. 2	Site No. 3
1.0 to 1.2	Mean	1.08	1.01	1.07
	SD	0.03	0.04	0.07
	CV(%)	2.66	3.58	6.77
	n	8	8	8
1.6 to 2.0	Mean	1.81	1.71	1.78
	SD	0.05	0.05	0.19
	CV(%)	2.88	2.78	10.50
	n	8	8	8
3.0 to 5.0	Mean	4.39	3.65	4.49
	SD	0.22	0.18	0.79
	CV(%)	4.99	4.93	17.64
	n	8	8	8
7.0 to 11	Mean	7.21	8.26	8.23
	SD	0.15	0.83	0.87
	CV(%)	2.07	10.10	10.55
	n	8	8	8

SD (Standard Deviation), CV(%) (Coefficient of Variation).

Table 9. Lot-to-Lot Reproducibility

Ratio of LSI HER-2/*neu* to CEP 17	Statistics	Lot No. 1	Lot No. 2	Lot No. 3	Lot No. 4
1.0 to 1.2	Mean	1.05	1.07	1.02	1.04
	SD	0.07	0.06	0.03	0.05
	CV(%)	6.48	6.06	3.21	4.87
	n	6	6	6	6
1.6 to 2.0	Mean	1.78	1.77	1.77	1.75
	SD	0.10	0.13	0.15	0.09
	CV(%)	5.65	7.49	8.54	5.07
	n	6	6	6	6
3.0 to 5.0	Mean	4.08	3.92	4.57	4.14
	SD	0.44	0.34	0.96	0.40
	CV(%)	10.78	8.74	20.92	9.56
	n	6	6	6	6
7.0 to 11	Mean	7.67	7.72	7.89	8.33
	SD	0.69	0.72	0.88	1.06
	CV(%)	8.97	9.36	11.16	12.68
	n	6	6	6	6

SD (Standard Deviation), CV(%) (Coefficient of Variation).

Table 10. Day-to-Day Reproducibility

Ratio of LSI HER-2/*neu* to CEP 17	Statistics	Assay Day No. 1	Assay Day No. 2	Assay Day No. 3	Assay Day No. 4
1.0 to 1.2	Mean	1.06	1.07	1.02	1.04
	SD	0.06	0.07	0.05	0.04
	CV(%)	5.65	6.61	4.58	4.03
	n	6	6	6	6
1.6 to 2.0	Mean	1.76	1.77	1.77	1.77
	SD	0.17	0.14	0.08	0.10
	CV(%)	9.62	7.99	4.31	5.65
	n	6	6	6	6
3.0 to 5.0	Mean	4.24	4.48	4.10	3.89
	SD	0.48	0.97	0.36	0.38
	CV(%)	11.25	21.56	8.89	9.71
	n	6	6	6	6
7.0 to 11	Mean	7.91	8.01	7.72	7.97
	SD	1.11	0.90	0.57	0.89
	CV(%)	13.99	11.22	7.39	11.20
	n	6	6	6	6

SD (Standard Deviation), CV(%) (Coefficient of Variation).

Table 11. Observer-to-Observer Reproducibility

Ratio of LSI HER-2/*neu* to CEP 17	Statistics	Observer No. 1	Observer No. 2
1.0 to 1.2	Mean	1.06	1.04
	SD	0.07	0.03
	CV(%)	7.00	2.85
	n	12	12
1.6 to 2.0	Mean	1.71	1.82
	SD	0.10	0.11
	CV(%)	6.01	6.20
	n	12	12
3.0 to 5.0	Mean	4.05	4.31
	SD	0.44	0.73
	CV(%)	10.80	16.84
	n	12	12
7.0 to 11	Mean	7.52	8.28
	SD	0.49	0.95
	CV(%)	6.55	11.44
	n	12	12

Assay Portability

A 5-center, blinded, randomized, comparative study using formalin-fixed, paraffin-embedded **human breast cancer specimens** was conducted to assess assay portability.[17] 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 1 normal (no amplification), 2 with low level and 1 with moderate level HER-2/*neu* gene amplification, as determined by FISH.

Day-to-Day Reproducibility

Table 12 shows the mean observed ratios of LSI HER-2/*neu* to CEP 17 for the 3 assay days. As shown by the *P*-values, there were no statistically significant variations in ratio values across the 3 study days (all *P*-values > 0.05). The results of this study demonstrated that the PathVysion assay is reproducible from day to day.

Table 12. Summary Statistics of LSI HER-2/*neu* to CEP 17 by Assay Day

Expected Ratio	Statistics	Assay Day No. 1	Assay Day No. 2	Assay Day No. 3	*P* value
1.0 to 1.2	Mean	1.01	1.06	1.05	0.6826
	SD	0.10	0.12	0.08	
	CV(%)	9.90	11.32	7.62	
	n	15	15	15	
2.1 to 2.8	Mean	2.54	2.43	2.32	0.5535
	SD	0.19	0.32	0.22	
	CV(%)	7.48	13.17	9.52	
	n	15	15	15	
2.5 to 3.5	Mean	3.18	2.98	3.03	0.2083
	SD	0.30	0.31	0.32	
	CV(%)	9.43	10.40	10.56	
	n	15	15	15	
5.0 to 7.0	Mean	5.69	5.63	5.69	0.9620
	SD	0.53	0.49	0.86	
	CV(%)	9.31	8.70	15.11	
	n	15	15	15	

Site-to-Site Reproducibility

Table 13 shows that the mean observed ratios of LSI HER-2/*neu* to CEP 17 for the 5 study sites. There was some statistically significant variation in the ratio value across the 5 study sites ($P = < 0.05$ for the normal and 2.5 to 3.5 specimen). However, these differences were not clinically relevant; 99% of the specimens were correctly classified as positive or negative for HER-2/*neu* gene amplification.

Table 13. Site-to-Site Reproducibility

Expected Ratio	Statistics	Site No. 1	Site No. 2	Site No. 3	Site No. 4	Site No. 5	*P* value
1.0 to 1.2	Mean	1.00	1.16	1.01	1.04	0.97	0.0001
	SD	0.09	0.09	0.07	0.09	0.04	
	CV(%)	9.00	7.76	6.93	8.65	4.12	
	n	9	9	9	9	9	
2.1 to 2.8	Mean	2.40	2.46	2.57	2.26	2.65	0.0965
	SD	0.19	0.26	0.52	0.22	0.24	
	CV(%)	7.92	10.60	20.20	9.73	9.06	
	n	9	9	9	9	9	
2.5 to 3.5	Mean	3.01	3.09	3.41	2.74	3.08	<0.0001
	SD	0.21	0.35	0.20	0.23	0.16	
	CV(%)	6.98	11.30	5.87	8.39	5.19	
	n	9	9	9	9	9	
5.0 to 7.0	Mean	5.48	5.22	5.94	5.82	5.91	0.0568
	SD	0.66	0.43	0.56	0.89	0.18	
	CV(%)	12.0	8.24	9.43	15.30	3.05	
	n	9	9	9	9	9	

The summary of assay variations for all 5 are presented in **Table 14**. The standard deviation (SD) and the coefficient of variation (CV) were relatively small and stable across all ratios of LSI HER-2/*neu* to CEP 17.

Table 14. Summary of Site-to-Site Reproducibility

Ratio of LSI HER-2/*neu* to CEP 17	Mean	Standard Deviation	CV (%)	n
1.0 to 1.2	1.04	0.10	9.60	45
2.1 to 2.8	2.47	0.32	12.96	45
2.5 to 3.5	3.07	0.31	10.10	45
5.0 to 7.0	5.67	0.63	11.11	45

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

Clinical Studies

Dose of Cyclophosphamide, Adriamycin and 5-Fluorouracil (CAF), CALGB 8869 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 3 different doses of adjuvant CAF chemotherapy: a high dose (cyclophosphamide at 600 mg/m^2, doxorubicin at 60 mg/m^2, and 5-fluorouracil at 600 mg/m^2 for 4 cycles), a moderate dose (cyclophosphamide at 400 mg/m^2, doxorubicin at 40 mg/m^2,and 5-fluorouracil at 400 mg/m^2 for 6 cycles) or a low dose (cyclophosphamide at 300 mg/m^2, doxorubicin at 30 mg/m^2, and 5-fluorouracil at 300 mg/m^2 for 4 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 PathVysion DNA Probe Kit, HER-2/*neu* gene amplification was defined as >2 (ie, 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.

Table 15 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 15. Comparison of Patient Characteristics at Baseline and Details of Adjuvant Treatment

	HER-2/*neu* amplification[a] n=91	No HER-2/*neu* amplification[a] n=433
Age		
<40	17.6	14.5
40 to 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 to ≤5	57.1	58.4
>5	9.9	3.9
unknown	1	0.5
Positive nodes		
≤3	59.3	55.9
4 to 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

[a] 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 16**). 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 16**)

Table 16. Cox Proportional Hazard Model Showing Likelihood-Ratio Tests for Disease-free and Overall Survival

	Disease-Free Survival			Overall Survival		
Source	**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 17, Figure 3a**) are comparable among the 3 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 17, 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 18, 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 18, Figure 3d**).

Table 17. Disease-free Survival Probabilities

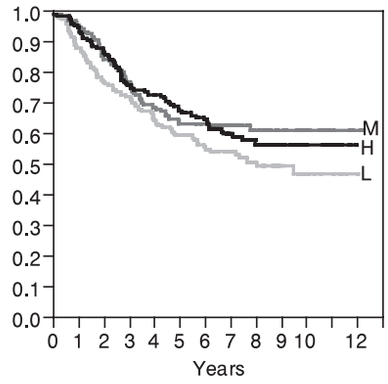
CAF Dose	HER-2/*neu* negative	HER-2/*neu* positive
Low	55%	36%
Moderate	63%	44%
High	61%	66%

Table 18. Overall Survival Probabilities

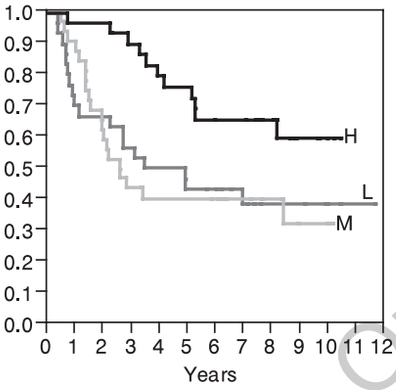
CAF Dose	HER-2/*neu* negative	HER-2/*neu* positive
Low	64%	48%
Moderate	75%	50%
High	70%	76%

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 3 CAF dose groups, H, M, and L[a]

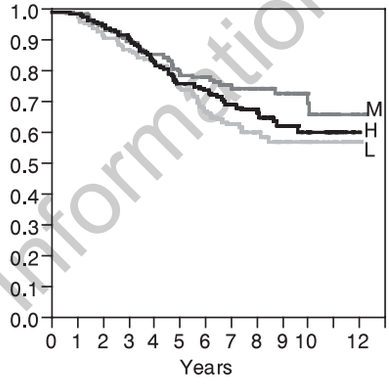
(a) Disease-free survival for HER-2/*neu*-negative



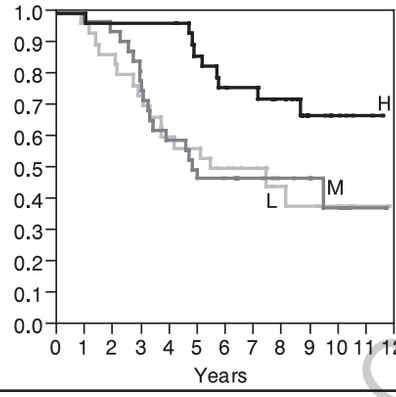
(b) Disease-free survival for HER-2/*neu*-positive



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



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



[a] 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 16**) 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.

Concordance with Clinical Trial Assay (CTA)

The primary mechanism of HER-2 protein overexpression in human breast cancer appears to be via gene amplification.[18-20] Fluorescence in situ hybridization (FISH) detection of HER-2/*neu* gene amplification provides an additional diagnostic method to define HER-2 overexpression.

The PathVysion kit was compared to the Clinical Trial Assay (CTA), which was used to enroll patients into the Genentech-sponsored pivotal HERCEPTIN trials (H0648g, H0649g, H0650g).[19] To establish concordance between FISH and the CTA, a subset of 623 specimens (317 positive and 306 negative, as determined by the CTA), were randomly selected in an intended 1:1 ratio from the specimens screened for enrollment in the HERCEPTIN trials. FISH assays were performed on all specimens, with informative results achieved on 529 specimens. The results from the analysis of the 529 informative cases are presented in **Table 19**.

Table 19. CTA versus FISH

	CTA Score				
FISH	0	1+	2+	3+	**Total**
Negative	207	28	67	21	323
Positive	7 (3.2%)	2 (6.7%)	21 (23.9%)	176 (89.3%)	206
Total	**214**	**30**	**88**	**197**	**529**

The results showed a 2 × 2 concordance of 82% (95% CI 79% to 85%), where concordance was defined as the proportion of samples rated 0 or 1+ by CTA and not amplified by FISH plus the proportion of samples rated 2+ or 3+ by CTA and amplified by FISH. These data are consistent with a high concordance between protein overexpression [as determined by immunohistochemistry (CTA)] and gene amplification [as determined by FISH (PathVysion Kit)].

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May 2016
30-608377/R7

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