

PATHVYSION HER-2 DNA Probe Kit II

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REF 06N46

30-608493/R8

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NOTE: CHANGES HIGHLIGHTED

Key to Symbols Used



Manufacturer



Reference Number



Lot Number



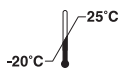
In Vitro Diagnostic Medical Device



Contains sufficient for <n> tests



Temperature Limitation



Temperature Limitation



Biological Risks



Danger



Danger



Danger



Danger



Consult instructions for use.



Use by



Authorized Representative in the European Community

PATHVYSION HER-2 DNA PROBE KIT II

List No. 06N46-030 (20 assays)/

List No. 06N46-035 (50 assays)/

List No. 06N46-036 (100 assays)

INTENDED USE

The PathVysion HER-2 DNA Probe Kit II (PathVysion Kit II) is designed to detect amplification of the HER-2/*neu* gene via fluorescence in situ hybridization (FISH) in formalin-fixed, paraffin-embedded human breast and gastric cancer tissue specimens.

For breast cancer indication, results from the PathVysion Kit II 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 II 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 II is indicated as an aid in the assessment of breast cancer patients for whom HERCEPTIN® (trastuzumab) treatment is being considered (see HERCEPTIN package insert).

Warning:

The following warning pertains to the PathVysion Kit II breast cancer indication only.

HERCEPTIN Therapy Selection

NOTE: All of the patients in the HERCEPTIN clinical trials were selected using an investigational immunohistochemical assay (clinical trial 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 assay 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 the HER-2/*neu* gene may still benefit from cyclophosphamide, doxorubicin, 5-fluorouracil (CAF) 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/*neu* 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

In 2008, breast cancer was the most common cancer (22.9%/1,384,155) and the most common cause of death from cancer (13.7%/458,503) worldwide among women.¹ Gastric cancer was the fourth most common cancer (9.7%/640,031) and the third most common cause of death in men (11.0%/463,930), as well as the fifth most common cancer (5.8%/348,571) and the fifth most common cause of death (8.2%/273,489) in women.¹

Breast Cancer

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 survival⁵ 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* (ERBB2) 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* status 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 hormone 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 primary breast carcinomas.

Gastric Cancer

Amplification or overexpression of the HER-2/*neu* gene has been shown in 22% of patients with advanced gastric cancer screened for HER-2/*neu* status.¹⁶

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 primary gastric carcinomas.

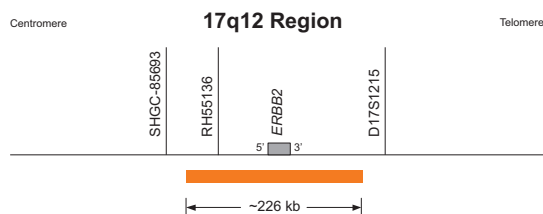
The FISH technique has been used to detect HER-2/*neu* gene amplification in human gastric carcinoma.¹⁶⁻¹⁹ In addition, the HER-2/*neu* status in primary and paired metastatic sites of gastric adenocarcinoma showed high concordance by FISH. This suggests that in gastric cancer HER-2/*neu* status is, in most cases, unchanged during the metastatic process.¹⁹

Principles of the Procedure

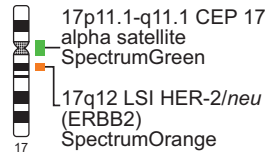
FISH is a technique that allows the visualization of specific nucleic acid sequences within a cellular preparation. Specifically, DNA FISH involves the precise annealing of a single-stranded, fluorescently-labeled DNA probe to complementary target sequences. The hybridization of the probe with the cellular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

The PathVysion HER-2 DNA Probe Kit consists of 2 fluorophore-labeled DNA probes in hybridization buffer with blocking DNA. The Vysis LSI HER-2/*neu* (ERBB2) SpectrumOrange Probe, approximately 226 Kb in length (chr17:37751152-37976854; February 2009 Assembly University of California, Santa Cruz [UCSC] Human Genome Browser) encompassing the HER-2/*neu* (ERBB2) gene located at chromosomal locus 17q12. The Chromosome Enumeration Probe (CEP) 17 DNA probe is an approximately 5.4 kb SpectrumGreen (SG) 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 premixed and predenatured 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. The PathVysion Kit II is designed for the detection of HER-2/*neu* gene amplification in formalin-fixed, paraffin-embedded (FFPE) human breast and gastric tissue specimens by FISH.

FFPE tissue specimens are placed on slides. The specimens are processed through a series of chemical and enzymatic pretreatment steps to increase the accessibility of the DNA for hybridization. The DNA is denatured to single-stranded form and subsequently allowed to hybridize with the 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 probes is viewed using a fluorescence microscope equipped with appropriate excitation and emission filters allowing visualization of the 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.



LSI HER-2/*neu* (ERBB2) SpectrumOrange Probe



WARNINGS AND PRECAUTIONS

IVD In Vitro Diagnostic Medical Device

- For In Vitro Diagnostic Use
- The PathVysion Kit II is intended for use only on FFPE breast and gastric cancer tissues; it is not intended for use on nonbreast, nongastric, or fresh cancer tissues.
- All biological specimens should be treated as if capable of transmitting infectious agents. The ProbeChek HER-2/*neu* 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.²¹
- 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.
- Failure to follow all procedures for slide denaturation, hybridization, and detection may cause unacceptable or erroneous results.
- To identify target areas, hematoxylin and eosin (H & E) staining should be conducted on every 10th slide of the same tissue block.
- Hybridization conditions may be adversely affected by the use of reagents other than those provided by Abbott Molecular.
- 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.
- If stored at low temperatures, 20X SSC may crystallize. If the crystals cannot be redissolved at room temperature, the solution should be discarded.
- If any other working reagents precipitate or become cloudy, they should be discarded and fresh solutions prepared.
- Fluorophores can be 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 that do not require light for manipulation (incubation periods, washes, etc) in the dark.
- The LSI HER-2/*neu* and CEP 17 DNA probe mixture contains formamide, which is a teratogen. Avoid contact with skin and mucous membranes.
- Calibrated thermometers are required for measuring temperatures of solutions, water baths, and incubators.
- 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.
- 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,²⁰ OSHA Standards on Bloodborne Pathogens,²¹ CLSI Document M29-A3,²² and other appropriate biosafety practices.²³ 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.²⁰
- Decontaminate and dispose of all potentially infectious materials in accordance with local, state, and federal regulations.²³

**PathVysion LSI HER-2/*neu* SpectrumOrange/CEP 17
SpectrumGreen Probes (continued)**

Hazard-determining components of labeling: Formamide



Danger

- H360 May damage fertility or the unborn child.
P201 Obtain special instructions before use.
P202 Do not handle until all safety precautions have been read and understood.
P280 Wear protective gloves/ protective clothing/ eye protection.
P308+P313 IF exposed or concerned:
Get medical advice/attention.
P405 Store locked up.
P501 Dispose of contents / container in accordance with local regulations.

NP-40

Hazard-determining components of labeling: Polyethylene glycol octylphenyl ether



Danger

- H302 Harmful if swallowed.
H315 Causes Skin Irritation.
H318 Causes serious eye damage.
H411 Toxic 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/ doctor if you feel unwell.
P501 Dispose of contents / container in accordance with local regulations.



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

REAGENTS AND INSTRUMENTS

Materials Provided

This kit contains sufficient reagents to process 20, 50, or 100 assays dependent on product ordered. An assay is defined as one 22 mm × 22 mm target area.

1. Vysis LSI HER-2/*neu* SpectrumOrange/CEP 17 SpectrumGreen Probes

- Part No.: 30-171062/35-171062
Quantity: 200 µL/500 µL/500 µL × 2 for the 100 assay kit
Composition: 20 ng/µL SpectrumGreen fluorophore-labeled alpha satellite DNA probe for chromosome 17, 10 ng/µL SpectrumOrange fluorophore-labeled DNA probe for the HER-2/*neu* gene locus and blocking DNA, predenatured in hybridization buffer.

2. DAPI Counterstain

- Part No.: 30-804840
Quantity: 300µL x 1/2/4 for the 20, 50, and 100 assay kit
Composition: 1000 ng/mL DAPI (4,6-diamidino-2-phenylindole) in phenylenediamine dihydrochloride, glycerol, and buffer.


3. NP-40

- Part No.: 30-804820
Quantity: 2000 µL x 2
Composition: Igepal (NP-40 substitute) (Octyl phenoxy) polyethoxyethanol.

4. 20X SSC

- Part No.: 30-805850
Quantity: 66 g
Composition: Sodium chloride and sodium citrate.

Storage and Handling

 -20°C Store the unopened PathVysion HER-2 DNA Probe Kit II as a unit at -20°C, protected from light.

The 20X SSC and NP-40 may be stored separately at room temperature (15 to 30°C). Expiration dates for each of the components are indicated on the individual component labels. These storage conditions apply to both opened and unopened components.

BREAST CANCER

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 (normal LSI HER-2/*neu* to 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 (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) Quantity: 5 × 25 mg
 - 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 (or equivalent; eg, d-Limonene)
- Hematoxylin and eosin (H & E)
- Immersion oil appropriate for fluorescence microscopy. Store at room temperature (15 to 30°C).
- Formamide, ultra pure
- Ethanol (100%). Store at room temperature.
- Concentrated (12N) hydrochloric acid (HCl)
- 1N sodium hydroxide (NaOH)
- Purified water (distilled, 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°C)
- Air incubators (37°C and 56°C)
- Diamond-tipped scribe
- Humidified hybridization chamber
- Forceps
- Disposable syringe (5 mL)
- Coplin jars (6). Suggested type: 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. The microscope should be checked to confirm 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 maintenance by the manufacturer's technical representative, especially alignment of the mercury lamp, are advised.

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 or other lamp with similar intensity and spectral output 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 or other lamp with similar intensity and spectral output. 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 autofluorescence and specifically for use in fluorescence microscopy.

Filters: Multi-bandpass fluorescence microscope filter sets optimized for use with the PathVysion Kit II are available from Abbott Molecular for most microscope models. The recommended filter sets for the PathVysion Kit II 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, add together:

66 g	20X SSC
200 mL	purified water

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 to 8.0)

To prepare, 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

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.

SPECIMEN PROCESSING AND SLIDE PREPARATION

Specimen Collection and Processing

The PathVysion Kit II is designed for use on FFPE tissue specimens. Tissue collections should be performed according to the laboratory's standard procedures. **Selection of tissue area for the PathVysion II 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 μm thick. FFPE tissue may be handled and stored according to the laboratory's routine procedures. To ensure optimum results from the PathVysion Kit II, 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 II contains reagents sufficient for approximately 20, 50, or 100 assays. One assay for the PathVysion Kit II is defined as a 22 mm \times 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 FFPE 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°C.
3. Mount the section on the positive side of an organosilane-coated slide.
4. Allow slides to air-dry.

(Start processing ProbeChek HER-2/*neu* 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 II. 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 clearing agent 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°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 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 Kit II assay protocol.

ASSAY PROCEDURE

FLUORESCENCE IN SITU HYBRIDIZATION

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 \times 3 in taped to the side of the container) to 37°C by placing it in the 37°C incubator prior to slide preparation. Moisten the blotting paper or paper towel with water before each use of the hybridization chamber.

2. Verify that the pH of the denaturing solution is 7.0 to 8.0 at room temperature before use. Add denaturing solution to Coplin jar and place in a 72 \pm 1°C water bath for at least 30 minutes or until the solution temperature reaches 72 \pm 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 \pm 1°C (\leq 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 \times 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°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 \pm 1°C water bath for at least 30 minutes or until solution temperature has reached 72 \pm 1°C.

NOTE: The temperature of the wash solution must return to 72 \pm 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 \pm 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 (Breast Cancer) in **Table 3** 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 nonspecificity or with noisy background. Skip nuclei with insufficient counterstain to determine the nuclear border. Enumerate only those nuclei with discrete signals.

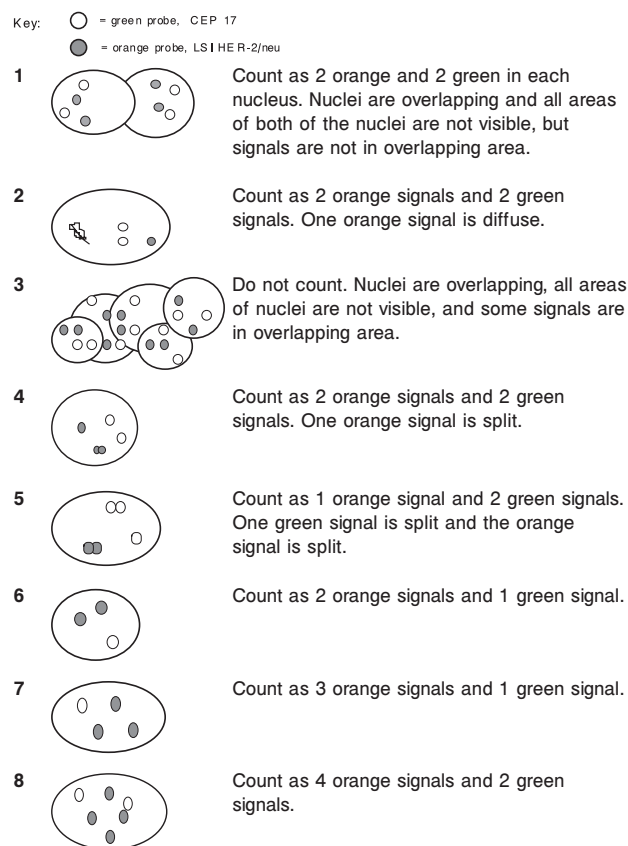
Signal Enumeration

Using a 40X objective, scan several areas of tumor cells to account for possible heterogeneity. Select an area of good nuclear 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 LSI HER-2/*neu* to CEP 17 Ratio Determination).

Figure 1. Dual Color Signal Counting Guide



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

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

1. Determine and record the number of LSI HER-2/*neu* and CEP 17 counts in 20 nuclei (see **Table 1** for an example).
2. Add all LSI HER-2/*neu* signals. This sum represents the total LSI HER-2/*neu* signals (eg, 252 in **Table 1**).
3. Add all CEP 17 signals. This sum represents the total CEP 17 signals (eg, 44 in **Table 1**).
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.

Table 1. Sample Data Sheet for Breast Cancer

Cell No.	LSI HER-2/ <i>neu</i>	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 II lot. ProbeChek HER-2/*neu* 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* to CEP 17 ratio results should be within the established ranges for acceptable test performance. See **Table 2** for acceptable ProbeChek HER-2/*neu* Control Slides 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 II 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 (Breast Cancer) in **Table 3**. 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 2. Acceptable HER-2/*neu* to CEP 17 Ratio Ranges for ProbeChek HER-2/*neu* Control Slides

Control	HER-2/ <i>neu</i> to 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 2A. Acceptable HER-2/*neu* to CEP 17 Ratio Ranges for Abbott AutoVysion System (Automated) Enumeration of ProbeChek HER-2/*neu* Control Slides

Control	HER-2/ <i>neu</i> to 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 3. Troubleshooting Guide (Breast Cancer)

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) or other lamp with similar intensity and spectral output.	• Replace with a new lamp.
		• Mercury lamp too old	• Realign lamp.
	• Mercury lamp misaligned	• Clean or replace lens.	
	• Dirty and/or cracked collector lenses	• Clean or replace mirror.	
	• Dirty or broken mirror in lamp house	• Check temperature of 37 ± 1°C incubator.	
	• Hybridization conditions inappropriate	• Increase hybridization time to at least 14 hours.	
	• Inappropriate post-hybridization wash temperature	• 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.	
	• Section overfixed (cell boundaries will be distinct)	• Check that pH of buffer is 2.0 ± 0.2.	
• DNA loss (poor DAPI staining)	• Increase digestion time, up to 60 min.	• 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.	• Check fixation conditions.	

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.
• Tissue loss or tissue morphology degraded	• Tissue section under fixed (poor DAPI staining)	• Provide gentle agitation during wash.
	• Inappropriate slides used	• Increase wash time to 5 minutes.
	• Improper slide baking	• Check fixation time/ conditions.
• Tissue section was torn removing coverslip after hybridization	• Overpretreatment	• Use positively charged slides.
	• Overdenaturation	• Check temperature of 56°C oven.
		• Check temperature of 80 ± 1°C pretreatment solution.
		• Decrease pretreatment time.
		• Decrease protease digestion time.
		• Check temperature of 72 ± 1°C denaturation solution.
		• Decrease denaturation time to 10 minutes.
		• Allow coverslip to soak off in post-hybridization wash buffer.

Contact Abbott Molecular Technical Services 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. The clinical study conducted found that specimens with amplification showed a LSI HER-2/*neu* to 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 (Breast Cancer) in **Table 3** 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.

- 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

- The PathVysion Kit II breast cancer assay has only been optimized for identifying and quantifying chromosome 17 and the *HER-2/neu* gene in interphase nuclei from FFPE human breast tissue specimens. Other types of specimens or fixatives should not be used.
- The performance of the PathVysion Kit II was validated using the procedures provided in this package insert only. Modifications to these procedures may alter the performance of the assay.
- Performance characteristics of the PathVysion Kit II 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.
- 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.
- FISH assay results may not be informative if the specimen quality and/or specimen slide preparation is inadequate.
- 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 Cutoff Point](#) 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 4](#). The distribution of ratios of *HER-2/neu* to CEP 17 signals for the 91 *HER-2/neu* amplified specimens is summarized in [Table 5](#).

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

Statistics	Range		
	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

SD = standard deviation

Table 5. Distribution of Ratio of LSI *HER-2/neu* to CEP 17 Signals in Amplified Breast Tissue Specimens

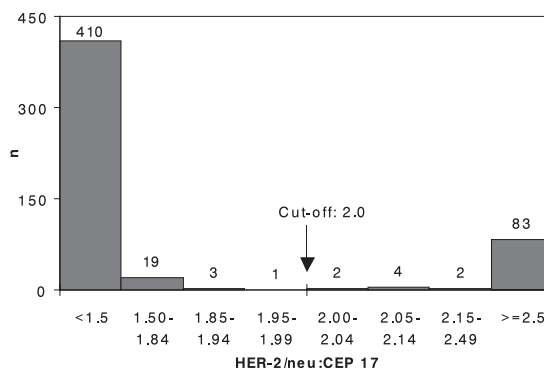
Statistics	Range		
	2.0 to 5.0	5.1 to 10.0	> 10.0
Mean	3.35	7.39	12.77
SD	0.95	1.41	1.80
n	33	42	16

SD = standard deviation

Establishment of Cutoff 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* to CEP 17 in the 524 specimens from the study described above are shown in [Figure 2](#).

Figure 2. Signal Distribution



PERFORMANCE CHARACTERISTICS

The following performance characteristics were determined using the PathVysion *HER-2* DNA Probe Kit (PathVysion Kit), List No. 02J01.

Analytical Sensitivity and Specificity

Hybridization Efficiency

On the ProbeChek *HER-2/neu* Control Slides, the average percentage of cells with no hybridization signal was 0.0 to 2.0%. These slides are prepared from FFPE 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* to CEP 17 ratio specimen was estimated with a mean of 1.05 (± 0.03) and the 1.6 to 2.0 *HER-2/neu* to 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 FFPE 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, 14, 18, 22, and 26 hours) 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 \pm 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 PathVysion 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). Results are shown in **Table 6**. 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). Results are shown in **Table 7**.

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

Table 6. Average Number of Signals per Cell and Ratio of LSI HER-2/*neu* to 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

Table 7. Average Number of Signals per Cell and Ratio of LSI HER-2/*neu* to 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

Table 8. Average Number of Signals per Cell and Ratio of LSI HER-2/*neu* to CEP 17 Copy Number in Consecutive Sections of Different Thickness

	Thickness of Section (μm)							
	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 PathVysion 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 FFPE 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 9** through **Table 12**.

Table 9. Site-to-Site Reproducibility

Ratio of LSI HER-2/ <i>neu</i> to CEP 17	Statistics	Site	Site	Site
		No. 1	No. 2	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 10. Lot-to-Lot Reproducibility

Ratio of LSI HER-2/ <i>neu</i> to CEP 17	Statistics	Lot	Lot	Lot	Lot
		No. 1	No. 2	No. 3	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 11. Day-to-Day Reproducibility

Ratio of LSI HER-2/ <i>neu</i> to CEP 17	Statistics	Assay	Assay	Assay	Assay
		Day No. 1	Day No. 2	Day No. 3	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 12. Observer-to-Observer Reproducibility

Ratio of LSI HER-2/ <i>neu</i> to CEP 17	Statistics	Observer	
		No. 1	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

SD = standard deviation; CV(%) = coefficient of variation

Assay Portability

A 5-center, blinded, randomized, comparative study using FFPE human breast cancer specimens was conducted to assess assay portability.²⁴ Study specimens consisted of FFPE 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 13 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 13. Summary Statistics of LSI HER-2/*neu* to CEP 17 by Assay Day

Expected Ratio	Statistics	Assay Day			<i>P</i> value
		Assay Day No. 1	Assay Day No. 2	Assay Day No. 3	
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	

SD = standard deviation; CV(%) = coefficient of variation

Site-to-Site Reproducibility

Table 14 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 14. Site-to-Site Reproducibility

Expected Ratio	Statistics	Site					<i>P</i> value
		Site No. 1	Site No. 2	Site No. 3	Site No. 4	Site No. 5	
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	

SD = standard deviation; CV(%) = coefficient of variation

The summary of assay variations for all 5 are presented in Table 15. 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 15. Summary of Site-to-Site Reproducibility

Ratio of LSI HER-2/ <i>neu</i> to CEP 17	Mean	SD	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

SD = standard deviation; CV(%) = coefficient of variation

This study showed a 100% hybridization success rate, validating the ease of use of the PathVysion Kit.

Clinical Studies

Dose of Cyclophosphamide, Adriamycin (doxorubicin), 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/m², doxorubicin at 60 mg/m², and 5-fluorouracil at 600 mg/m² for 4 cycles),
- A moderate dose (cyclophosphamide at 400 mg/m², doxorubicin at 40 mg/m², and 5-fluorouracil at 400 mg/m² for 6 cycles), or
- A low dose (cyclophosphamide at 300 mg/m², doxorubicin at 30 mg/m², and 5-fluorouracil at 300 mg/m² 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 assay, 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 16 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 16. Comparison of Patient Characteristics at Baseline and Details of Adjuvant Treatment

	HER-2/ <i>neu</i> amplification* n=91	No HER-2/ <i>neu</i> amplification* n=433
<u>Age</u>		
<40	17.6	14.5
40 to 50	39.6	40.0
>50	42.9	48.5
<u>Menopausal status</u>		
Premenopausal	46.2	39.5
Peri/Postmenopausal	53.8	60.5
<u>Tumor size</u>		
≤2 cm	31.9	37.2
>2 to ≤5	57.1	58.4
>5	9.9	3.9
unknown	1	0.5
<u>Positive nodes</u>		
≤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
<u>CAF dose regimen received</u>		
High	33.0	34.4
Moderate	34.1	31.4
Low	33.0	34.2

* Percent of patients
ER=estrogen; PR=progesterone

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 17**). 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 17**).

Table 17. 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

DF = degrees of freedom; ChiSq = chi-square

Disease-free survival probabilities (**Table 18, 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 18, 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 19, 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 19, Figure 3d**).

Table 18. Disease-free Survival Probabilities

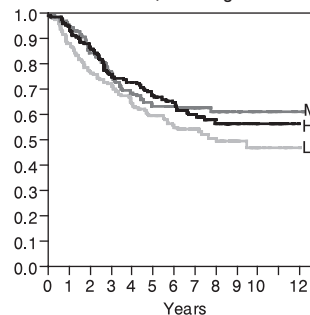
CAF Dose	HER-2/ <i>neu</i> -negative	HER-2/ <i>neu</i> -positive
Low	55%	36%
Moderate	63%	44%
High	61%	66%

Table 19. Overall Survival Probabilities

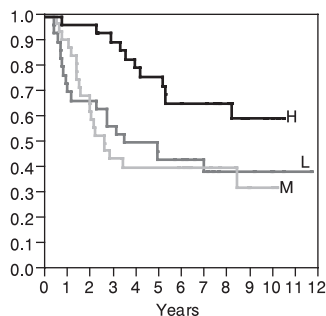
CAF Dose	HER-2/ <i>neu</i> -negative	HER-2/ <i>neu</i> -positive
Low	64%	48%
Moderate	75%	50%
High	70%	76%

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 3 CAF dose groups, H, M, and L*

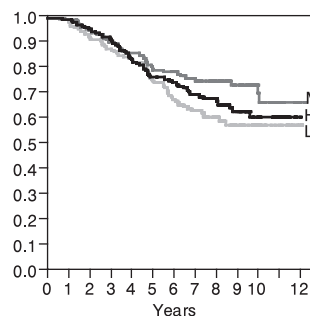
(a) Disease-free survival for HER-2/*neu*-negative



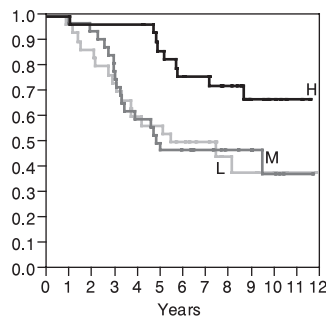
(b) Disease-free survival for HER-2/*neu*-positive



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



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



*HER-2/*neu* positivity means HER/CEP ≥ 2. Sample sizes in (a, c) are 149, 136, and 148 (for H, M, and L) and in (b, d) are 30, 31, and 30. The significance levels for the HER-2/*neu* by CAF interaction from the proportional hazards models (**Table 17**) 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/*neu* protein overexpression in human breast cancer appears to be via gene amplification.²⁵⁻²⁸ Fluorescence in situ hybridization (FISH) detection of HER-2/*neu* gene amplification provides an additional diagnostic method to define HER-2/*neu* overexpression.

The PathVysion Kit was compared to the CTA, which was used to enroll patients into the Genentech-sponsored pivotal HERCEPTIN trials (H0648g, H0649g, and H0650g).^{26,27} 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 20**.

Table 20. CTA versus FISH

FISH	CTA Score				Total
	0	1+	2+	3+	
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]).

GASTRIC CANCER

Materials Required But Not Provided

Laboratory Reagents

- Gastric Pretreatment Reagents (manual assay)
 - Protease IV, 75 mg (List No. 06N46-001)
- Gastric Pretreatment Reagents (automated assay — VP 2000)
 - Protease IV, 750 mg (List No. 06N46-002)
 - 20X SSC: 500 g (List No. 02J10-032)
- Hemo-De clearing agent (or equivalent; eg, d-Limonene)
- Hematoxylin and eosin (H & E)
- Immersion oil appropriate for fluorescence microscopy. Store at room temperature (15 to 30°C).
- Ethanol (100%). Store at room temperature.
- Concentrated (12N) hydrochloric acid (HCl)
- 1N HCl
- Sodium hydroxide (NaOH)
- Purified water (distilled, deionized, or Milli-Q). Store at room temperature.
- Rubber cement

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

Laboratory Equipment

- VP 2000 Processor
- ThermoBrite (List No. 07J91-010 or 07J91-020)
- ThermoBrite humidity cards (List No. 07J68-001)
- Precleaned silanized or positively-charged glass microscope slides
- 22 mm × 22 mm glass coverslips
- Microliter pipettor (1 to 10 µL) and sterile tips
- Timer
- Microtome
- Microcentrifuge
- Magnetic stirrer
- Vortex mixer
- Graduated cylinder
- Circulating water baths (73°C and 80°C)

NOTE: Static water baths do not provide adequate temperature control for higher temperature baths.

- Circulating or non-circulating water bath (37°C)
- Protein-free water bath (40 ± 2°C)
- Air incubators/oven/hot plate (56 ± 2°C)
- Glass scribe
- Forceps
- Disposable syringe (5 mL)
- Coplin jars (50 to 70 mL); Suggested type: vertical staining jar
- Fluorescence microscope equipped with recommended filters (see next section)
- pH meter
- Calibrated thermometer
- Microscope slide box with lid
- 0.22 or 0.45 µm pore filtration unit

Microscope Equipment and Accessories

Microscope: An epi-illumination fluorescence microscope is required for viewing the hybridization results. The microscope should be checked to confirm 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 maintenance by the manufacturer's technical representative, especially alignment of the mercury lamp, are advised.

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 or other lamp with similar intensity and spectral output 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 or other lamp with similar intensity and spectral output. 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 autofluorescence and specifically for use in fluorescence microscopy.

Filters: Single-bandpass fluorescence microscope filter sets optimized for use with the PathVysion Kit II are available from Abbott Molecular for most microscope models. The recommended filter sets for the PathVysion Kit II are the Orange single-bandpass, Green single-bandpass, and DAPI single-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.

Using this Vysis filter	Allows excitation and emission of...
Orange single-bandpass	SpectrumOrange fluorophore
Green single-bandpass	SpectrumGreen fluorophore
DAPI single-bandpass	DAPI

Preparation of Stock Reagents

NOTE: Where indicated, measure the pH of these solutions at room temperature. Use a pH meter with a glass electrode unless otherwise noted.

20X SSC Solution (250 mL for manual assay pretreatment)

To prepare, add together:

66 g	20X SSC
200 mL	purified water

Mix thoroughly. Bring the total volume to 250 mL with purified water. Filter through a 0.22 or 0.45 µm pore filtration unit. Store at room temperature for up to 6 months.

20X SSC Solution (500 mL for automated assay — VP 2000 pretreatment)

To prepare, add together:

132 g	20X SSC
400 mL	purified water

Mix thoroughly. Bring the total volume to 500 mL with purified water. Filter through a 0.22 or 0.45 µm pore filtration unit. Store at room temperature for up to 6 months.

1X SSC Pretreatment Solution (1 L)

To prepare, add together:

50 mL	20X SSC Solution
900 mL	purified water

Mix thoroughly. Measure pH and adjust to pH 6.3 ± 0.2 with NaOH or HCl. Add purified water to bring final volume to 1 liter. Filter through a 0.22 or 0.45 µm pore filtration unit. Store at room temperature for up to 6 months.

Ethanol Solutions

Prepare v/v dilutions of 70%, 85%, and 100%, using 100% ethanol and purified water. Store at room temperature in tightly capped containers when not in use.

Post-Hybridization Wash Buffer (2X SSC/0.3% NP-40) (1 L)

To prepare, add together:

100 mL	20X SSC
850 mL	purified water
3 mL	NP-40

Mix thoroughly. Measure pH at room temperature using a pH meter. Adjust pH to 7.0 to 7.5 with NaOH or HCl. Adjust volume to 1 liter with purified water. Filter through 0.22 or 0.45 µm pore filtration unit. Store unused solution at room temperature for up to 6 months.

0.1N HCl Protease Buffer (1L)

To prepare, add together:

100 mL	1N HCl
900 mL	purified water

Mix thoroughly. Store unused solution at room temperature for up to 6 months.

Preparation of Working Reagents for Manual Procedure

NOTE: The volumes of solutions are given for the 50 to 70 mL Coplin jars (see Laboratory Equipment). Larger volumes may be required when using different Coplin jars. Enough solution should be used to cover the target area, but not so much as to immerse the slide labels.

Hemo-De Clearing Agent

Fill 3 Coplin jars with 50 to 70 mL of Hemo-De clearing agent. Discard after 1 week of use.

Pretreatment Solution

Pour 50 to 70 mL of 1X SSC pH 6.3 into a Coplin jar. Warm the jar from room temperature to 80±1°C in a circulating water bath prior to pretreating the slides. Ensure that the temperature of the solution is 80±1°C before using. Discard after 1 day of use.

Protease Solution (1.5 mg/mL)

Add 75 mg Protease (List No. 06N46-001) to 50 mL of 0.1 N HCl for a final concentration of 1.5 mg/mL Protease. Gently mix. Place the protease solution into a Coplin jar, and place the Coplin jar in a 37±1°C water bath. Allow a minimum of 30 minutes with intermittent mixing before starting the assay and ensure that the temperature of the buffer is 37±1°C before use. Visually verify protease is completely dissolved before use. Discard solution after 1 day of use.

Purified Water

Fill 1 Coplin jar with 50 to 70 mL of purified water. Use at room temperature.

Ethanol Solutions

Solutions may be used for 1 week unless evaporation occurs or the solution becomes diluted due to excessive use.

Fill 3 Coplin jars with 50 to 70 mL 100% ethanol.

Fill 1 jar with 50 to 70 mL 85% ethanol.

Fill 1 jar with 50 to 70 mL 70% ethanol.

Preparation of Working Reagents for VP 2000 Procedure

Hemo-De Clearing Agent

Fill 3 VP 2000 basins (room temperature) with 500 mL each Hemo-De clearing agent. Discard after 1 week of use.

Pretreatment Solution

Pour 500 mL of 1X SSC pH 6.3 into the VP 2000 basin (heated). Warm the solution from room temperature to 80°C prior to pretreating the slides. Ensure that the temperature of the solution is 80°C before using. Discard after 1 day of use.

Protease Solution (1.5 mg/mL)

Add 750 mg Protease (List No. 06N46-002) to 500 mL of 0.1N HCl in the VP 2000 basin for a final concentration of 1.5 mg/mL. Mix gently and heat to 37±1°C. Allow a minimum of 30 minutes with intermittent mixing before starting the assay and ensure that the temperature of the buffer is 37±1°C before use. Visually verify protease is completely dissolved before use. Discard solution after 1 day of use.

Purified Water

VP 2000 should be connected to a purified water source to support the continuous-flow rinse basin.

Ethanol Solutions

Solutions may be used for 1 week unless evaporation occurs or the solution becomes diluted due to excessive use.

Fill 3 VP 2000 basins (room temperature) with 500 mL 100% ethanol.

Fill 1 VP 2000 basin (room temperature) with 500 mL 85% ethanol.

Fill 1 VP 2000 basin (room temperature) with 500 mL each 70% ethanol.

SPECIMEN PROCESSING AND SLIDE PREPARATION

Specimen Collection and Processing

The PathVysion Kit II is designed for use on paraffin-embedded gastric cancer tissue specimens that are fixed in formalin (10% neutral buffered formalin). Tissue collections should be performed according to the laboratory's standard procedures. 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.

Gastric tissue should be prepared in sections between 4 and 6 µm thick. FFPE tissue may be handled and stored according to the laboratory's routine procedures. To ensure optimum results from the PathVysion Kit II, these methods should be consistent for all specimens analyzed.

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 II contains reagents sufficient for approximately 20, 50, or 100 assays. One assay for the PathVysion Kit II 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

1. Cut 5±1 µm thick paraffin sections using a microtome.

2. Float the sections in a protein-free water bath at 40±2°C.
3. Mount a section on a positively-charged slide.
4. Air-dry the slides overnight.
5. Perform conventional H & E staining of 1 specimen slide.

NOTE: The specimen slide used for the assay procedure should be within 10 serial sections of the H & E slide.

NOTE: Step 6 should be performed by a pathologist.

6. Examine and mark (encircle) the largest possible area of tumor, excluding necrotic, inflammatory, or normal mucosa.
7. With a glass scribe, transfer the mark from the H & E slide to the corresponding areas of the unstained slide, marking the glass on the side opposite the tissue section.
8. Bake the unstained slides 2 to 24 hours at 56±2°C.
9. Process the unstained slides following the procedures below.

Slide Pretreatment

Manual Pretreatment

Deparaffinizing Slides

1. Immerse slides in Hemo-De clearing agent for 5 minutes at room temperature.
2. Repeat step 1, using fresh Hemo-De.
3. Repeat step 1 again, using fresh Hemo-De.
4. Dehydrate slides in 100% ethanol for 1 minute at room temperature.
5. Repeat step 4. Proceed immediately to the slide pretreatment.

Pretreating Slides

6. Immerse slides in pretreatment solution (1X SSC; pH 6.3) at 80±1°C for 35±5 minutes. If necessary, 2 slides may be placed back-to-back in each slot in the Coplin jar, with 1 slide placed in each end slot. For the end slides, the side of the slide with the tissue section must face the center of the jar.
7. Immerse slides in purified water for 3 minutes at room temperature. Remove slides from the jar of purified water. Remove excess water by blotting the edges of the slides on a paper towel; do not allow to dry.

Protease Treatment

8. Immerse slides in protease solution (Protease IV, [List No. 06N46-001] 1.5 mg/mL in 0.1N HCl) at 37±1°C for 20±3 minutes. If necessary, 2 slides may be placed back-to-back in each slot in the Coplin jar, with 1 slide placed in each end slot. For the end slides, the side of the slide with the tissue section must face the center of the jar.
9. Immerse slides in purified water for 3 minutes at room temperature.
10. Immerse the slides in 70% ethanol for 1 minute.
11. Immerse the slides in 85% ethanol for 1 minute.
12. Immerse the slides in 100% ethanol for 1 minute.
13. Air-dry slides or dry slides on a slide warmer.
Proceed to Co-Denaturation/Hybridization of Specimen DNA procedure.

Automated (VP 2000) Pretreatment

See the Abbott VP 2000 Processor Operations Manual for more information on how to use the instrument. The VP 2000 pretreatment protocol is provided in **Table 21**.

NOTE: Use Protease IV (List No. 06N46-002), 1.5 mg/mL in 0.1N HCl.

Table 21. VP 2000 Pretreatment Protocol

Step	Solution	Temperature	Time
1	Hemo-De	Room	5 minutes
2	Hemo-De	Room	5 minutes
3	Hemo-De	Room	5 minutes
4	100% Ethanol	Room	1 minute
5	100% Ethanol	Room	1 minute
6	1X SSC Pretreatment Solution	80°C	35±5 min
7	Purified water, continuous flow setting	Room	3 minutes
8	Protease Solution	37°C	20±3 min
9	Purified water, continuous flow setting	Room	3 minutes
10	70% Ethanol	Room	1 minute
11	85% Ethanol	Room	1 minute
12	100% Ethanol	Room	1 minute
13	Air-dry		

Proceed to Co-Denaturation/Hybridization of Specimen DNA procedure.

ASSAY PROCEDURE

FLUORESCENCE IN SITU HYBRIDIZATION

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.

Co-Denaturation/Hybridization of Specimen DNA

See the ThermoBrite User Guide for more information on how to use the instrument.

1. Moisten a humidity card with water and place in the card slots of the ThermoBrite.
2. Set the Melt Temp to 73°C and the Melt Time to 5 minutes. Set the Hyb Temp to 37°C and the Hyb Time to 12 to 24 hours.
3. Apply 10 µL of probe mixture to a slide and immediately apply coverslip.
4. Seal edges of the coverslip with rubber cement.
5. Begin the co-denaturation/hybridization program.

At the end of the hybridization period (12 hours to 24 hours), 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 73 ± 1°C water bath for at least 30 minutes or until solution temperature has reached 73 ± 1°C.

NOTE: Measure the temperature of the wash solution by placing a temperature probe in the Coplin jar. The temperature of the wash solution must return to 73 ± 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 slides 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 and immerse slide in post-hybridization wash buffer at 73 ± 1°C for 2 minutes (4 slides/jar).

NOTE: It is recommended that 4 slides are processed per Coplin jar to maintain appropriate temperatures of the solutions. If you have less than 4 slides, add blank slides that are at room temperature to bring the total to 4.

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 signal enumeration.

Signal Enumeration

Assessing Slide Adequacy

Evaluate slide adequacy using the following criteria:

- Nuclear morphology: Examine the selected regions using a 40X to 63X objective, observing nuclear morphology in the scribed target area. Select well-digested areas of tissue for evaluation. Nuclei observed by DAPI should be distinguishable and have well-defined borders.
- Probe signal intensity: The signals should be bright, distinct, and easily evaluable. Signals should be compact oval shapes or stringy (diffuse) oval shapes.
- Background: The background should appear dark (black) and relatively free of fluorescence particles.

If the scribed target area on the slide does not meet these criteria, repeat the assay with a new slide. Assay conditions may be changed within the ranges specified in the Specimen Collection and Processing, Slide Pretreatment, Co-Denaturation/Hybridization of Specimen DNA, and Post-Hybridization Washes sections.

Selection of Optimum Viewing Area and Evaluable Nuclei

- Use a 10 to 25X objective and the DAPI single-bandpass filter to view the hybridized area and locate the target of interest (tumor cells as identified by H & E stain).
- The H & E slide used as a reference for scribing may be used for reference in selecting the optimum viewing area.
- Avoid areas of necrosis and where the nuclear borders are ambiguous.

Recognition of Target Signals

Definition of HER-2/*neu* amplification pattern: HER-2/*neu* amplification patterns often consist of signal clusters (see **Figure 4**, row 8) but may also consist of individual HER-2/*neu* signals in high copy number as compared to the CEP 17 copy number (see **Figure 4**, row 9).

- Scan the scribed target area using the prescribed filters and a 40X to 63X objective. The H & E slide used as a reference for scribing may be used for reference while evaluating signal distribution.
- Adjust the depth of focus to become familiar with size and shape of the target signals and noise (debris).
- Avoid nuclei that do not meet adequacy criteria as described in the Assessing Slide Adequacy section.
- Evaluate signal distribution only in tumor cells using the orange and green single-bandpass filters. In general, tumor cells are larger than normal cells, lymphocytes, and epithelial cells.
- Observe the overall signal distribution, which may be homogeneous or heterogeneous. In case of heterogeneous distribution, choose areas containing amplification patterns for enumeration.

Signal Enumeration

- Select approximately 4 non-overlapping fields of view for enumeration.
 - Homogeneous signal distribution: Choose approximately 4 fields of view containing tumor cells within the scribed target area for enumeration.
 - Heterogeneous signal distribution: If focal amplification patterns exist, choose approximately 4 fields of view containing amplified cells for enumeration. If amplified cells are intermixed with polysomal or disomal cells, choose approximately 4 fields of view that contain amplification patterns for enumeration, but include both amplified cells and consecutive nonamplified cells in the count of 20 cells.
- Using a 63X or 100X objective and DAPI single-bandpass filter, begin analysis in the upper left quadrant of the selected area. For each field view, choose approximately 5 consecutive nuclei that have intact, well-defined nuclei and clear nuclear boundaries for enumeration, then move to the next representative microscope field and repeat until a total of 20 nuclei have been enumerated. Switch to orange and green single-bandpass filters to count the number of signals within the nuclear boundary of each selected nucleus according to the guidelines provided below and in **Figure 4**.
- 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 size of 1 signal diameter as 1 signal (See **Figure 4**, row 4).
- Signal clusters are localized aggregations of signals containing a minimum of 3 adjacent signals separated by a distance less than the size of 1 signal diameter.
 - Individual signals may be difficult to enumerate due to close proximity to each other. In this case, an estimate of the signal copy number is made (See **Figure 4**, row 8).
 - Estimate the size of a single signal and then extrapolate this to the area containing the signal cluster. Estimate the area size in increments of 5 to 10, as recommended in **Table 22**.
 - If the nucleus contains individual LSI HER-2/*neu* signals outside of the signal cluster, include these in the signal estimate.
- 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. Record counts for each nucleus (refer to **Table 23**).
- When individual signals can be easily distinguished, record the actual count according to **Table 22**. When signal clusters are present, record an estimate of the signal number in increments of 5 and record the midpoint values (see recommendations in **Table 22**). These midpoint values should be used to calculate the LSI HER-2/*neu* to CEP 17 ratio (as exemplified in **Table 23**).

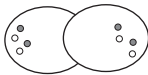
Table 22. Recommendation of Signal Number Estimation for Signal Clusters

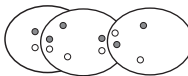
If the signal estimate falls in this range...	...Then record these values
Up to 10	Record estimated individual integer value
10 to 15	12.5
15 to 20	17.5
20 to 25	22.5
25 to 30	27.5
30 to 35	32.5
35 to 40	37.5
40 to 45	42.5
45 to 50	47.5
Over 50	50


Figure 4. Dual Color Signal Counting Guide


Key: ○ = green probe, CEP 17


● = orange probe, LSI HER-2/neu


- 


Count as 2 orange and 2 green signals in each nucleus. Nuclei are overlapping and all areas of both of the nuclei are not visible, but signals are not in overlapping area.
- 

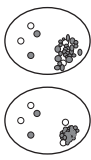
Do not count. Nuclei are overlapping, all areas of nuclei are not visible, and some signals are in overlapping area.
- 


Count as 2 orange signals and 2 green signals. One green signal is diffuse.
- 

Count as 2 orange signals and 2 green signals. One green signal is split and 1 orange signal is split.
- 

Count as 1 orange signal and 2 green signals.
- 

Count as 2 orange signals and 2 green signals.
- 

Count as 4 orange signals and 4 green signals.
- 

Signals in these nuclei are representative of a HER-2/neu amplification pattern. Estimate the number of orange (LSI HER-2/neu) signals in the cluster and add to the individual orange signals to obtain the total number of orange signals. Estimation of the number of LSI HER-2/neu signals in these examples is 25 to 30 and the number of CEP 17 signals is 4. See **Table 22** for more extensive explanation.
- 

Signals in this nucleus are representative of a HER-2/neu amplification pattern. Count as 5 orange (LSI HER-2/neu) signals and 2 green (CEP 17) signals.

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

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

- The numbers of LSI HER-2/neu and CEP 17 signals per nucleus are recorded in columns (refer to **Table 23** for an example).

Table 23. Sample Data Sheet for Gastric Cancer

Cell No.	LSI HER-2/neu	CEP 17
1	5	1
2	6	1
3	10	1
4	12.5	1
5	12.5	1
6	17.5	1
7	6	2
8	12.5	2
9	12.5	2
10	12.5	2
11	17.5	2
12	17.5	2
13	8	3
14	10	3
15	12.5	3
16	12.5	3
17	17.5	3
18	17.5	3
19	12.5	4
20	17.5	4
Total	250	44

- Add all LSI HER-2/neu signals (20 cells). This sum represents the total number of LSI HER-2/neu signals (eg, 250 in **Table 23**).
- Add all CEP 17 signals (20 cells). This sum represents the total number of CEP 17 signals (eg, 44 in **Table 23**).
- The LSI HER-2/neu to CEP 17 ratio is calculated by dividing the sum of LSI HER-2/neu signals by the sum of CEP 17 signals (eg, 250/44 equals a ratio of 5.68, which is positive for HER-2/neu amplification).
- If the ratio is > 2.2 or < 1.8 , then NO more enumeration is required. The final HER-2/neu amplification status will be based on this result. Go to step 7 for the final result.
- If the ratio is at or near the cutoff point (1.8 to 2.2), count an additional 20 nuclei and recalculate the ratio based on the combined sums for LSI HER-2/neu and CEP 17 signals (40 cells).
- When the final result is obtained:
If the ratio is < 2.0 , HER-2/neu gene amplification was not observed.
If the ratio is ≥ 2.0 , HER-2/neu gene amplification was observed.

QUALITY CONTROL

Assess slide adequacy according to the instructions in the **Signal Enumeration** section. The criteria for slide adequacy must be satisfied. Hybridization quality and efficiency should be considered when evaluating results.

Normal cells provide an internal control of assay quality.

- Normal cells contain 1 to 2 orange (LSI HER-2/neu) and 1 to 2 green (CEP 17) signals.
- Note that some normal cells have fewer than 2 signals of each color per cell due to truncation during the sectioning procedure.
- Normal areas of the slide should meet the criteria for slide adequacy.

The test is uninformative when interpretation of the hybridization signal is difficult or if there are insufficient cells for analysis. In the event of hybridization failure, consult the Troubleshooting Guide (Gastric Cancer) in **Table 24**.

Table 24. Troubleshooting Guide (Gastric Cancer)

Problem	Probable Cause	Solution
• No signal or weak signal	• Inappropriate filter set used to view slides • Microscope not functioning properly • Improper lamps (ie, Xenon or Tungsten) • Mercury lamp too old • Mercury lamp misaligned • Dirty or cracked collector lenses • Dirty or broken mirror in lamp house • Hybridization conditions inappropriate. • Inappropriate post-hybridization wash temperature • Air bubbles trapped under coverslip and prevented probe access • Insufficient amount of hybridization solution for section. • Inadequate protease digestion	• Use recommended filters. • Call microscope manufacturer's technical representative. • Use a mercury lamp (100-watt recommended) or other lamp with similar intensity and spectral output. • Replace with a new lamp. • Realign lamp. • Clean or replace lens. • Clean or replace mirror. • Increase hybridization time within the allowed range. • Verify temperature of the post-hybridization wash buffer. • Decrease the post-hybridization wash buffer temperature to the lower end of the allowed temperature range. • Apply coverslip by first touching the surface of the hybridization mixture. • Increase amount of hybridization solution to 20 µL per section. • Verify temperature of protease solution. • Verify protease used is Protease IV (List No. 06N46-001 or 06N46-002). • Verify that concentration of HCl is 0.1N HCl. • Verify protease concentration of 1.5 mg/mL in 0.1N HCl. • Increase protease digestion time within the allowed range.
• No signal or weak signal (continued)	• Inadequate pretreatment • Section overfixed (cell boundaries will be distinct)	• Verify temperature of pretreatment solution. • Verify pretreatment solution is 1X SSC, pH 6.3±0.2. • Increase pretreatment solution incubation time and/or temperature within the allowed range. • Prolonged tissue fixation times prior to paraffin embedding may lead to progressive degradation of signal intensity and may require increased protease digestion time or pretreatment incubation time/temperature. Increase protease digestion time or pretreatment solution incubation time and/or temperature within the allowed range.

Problem	Probable Cause	Solution
• Variation of signal intensity across tissue section	• Inherent in many tissue sections • Probe unevenly distributed on slide due to air bubbles under coverslip • Oversized section	• Check DAPI staining and signal intensity in target area. If majority of the target area has intact nuclei and bright signals, then score slide. • Repeat hybridization on next adjacent section of same tissue block and make sure no air bubbles are trapped under coverslip. • Apply coverslip by first touching the surface of the hybridization mixture. • Increase volume of hybridization probe mixture to 20 µL on large tissue sections.
• Noisy background	• Inadequate wash stringency	• Verify pH of post-hybridization wash solution is 7.0 to 7.5. • Verify temperature of post-hybridization wash buffer. • Increase post-hybridization wash buffer temperature to the upper end of allowed temperature range.
• Tissue loss or tissue morphology degraded	• Inappropriate slides used • Improper slide baking • Overpretreatment • Overdigestion • Overdenaturation • Tissue section was torn when removing coverslip after hybridization • Tissue section under fixed (poor DAPI staining)	• Use positively-charged slides. • Verify baking temperature and time. • If the paraffin on the slides does not appear to be melted, increase temperature and/or time within the allowed range. • Verify temperature of pretreatment solution. • Decrease time and/ or temperature within allowed range. • Decrease protease digestion incubation time within the allowed range. • Verify time and temperature of ThermoBrite denaturation. • Allow coverslip to soak off in post-hybridization wash buffer. • Shortened tissue fixation times prior to paraffin embedding may lead to excessive digestion and/or pretreatment of the sample. Decrease protease digestion time or pretreatment solution incubation time and/ or temperature within allowed range.

Contact Abbott Molecular Technical Services 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. Amplification is defined as an LSI HER-2/*neu* to CEP 17 signal ratio of ≥2.0; nonamplification is defined as 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.

Reasons to Repeat the Assay

The following are situations requiring repeat assays with fresh specimen slides. Consult the Troubleshooting Guide (Gastric Cancer) in **Table 24** for probable causes and the actions needed to correct specific problems.

1. If there are fewer than 20 evaluable nuclei, the test is uninformative and the assay should be repeated.
2. If any of the criteria for slide quality (nuclear morphology, signal intensity and background) are unsatisfactory as described in the Signal Enumeration section, the assay must be repeated.

LIMITATIONS

1. The PathVysion Kit II gastric cancer assay has only been optimized for identifying and quantifying chromosome 17 and the HER-2/*neu* gene in interphase nuclei from formalin-fixed, paraffin-embedded human gastric tissue specimens. Other types of specimens or fixatives should not be used.
2. The performance of the PathVysion Kit II was validated using the procedures provided in this package insert only. Modifications to these procedures may alter the performance of the assay.
3. FISH assay results may not be informative if the specimen quality and/or specimen slide preparation is inadequate.
4. Technologists performing the FISH signal enumeration must be capable of visually distinguishing between the orange and green signals.

PERFORMANCE CHARACTERISTICS

Success Rate

The success rate of the PathVysion Kit II assay was evaluated for both manual and VP 2000 pretreatment procedures. The success rate is defined as the percent of informative specimens out of the total number of specimens tested. Using manual pretreatment, 19 FFPE gastric cancer specimens (4 HER-2/*neu*-positive and 15 HER-2/*neu*-negative, as determined by an immunohistochemistry [IHC] assay) were tested in replicates of 4, for a total of 76 slides. Using VP 2000 pretreatment, the 19 specimens were tested in replicates of 2, for a total of 38 slides. Each slide was evaluated for signal intensity, signal specificity, slide background, and overall quality to determine performance of the PathVysion Kit II.

For the manual pretreatment procedure, the assay success rate was 100% (96.1%, lower-bound 95% CI). For the VP 2000 pretreatment procedure, the assay success rate was 100% (92.4%, lower-bound 95% CI). Results are shown in **Table 25**.

Table 25. Success Rate

Procedure	No. of Slides Tested	No. of Slides Passed	Assay Success Rate (%)	Lower Bound 95% CI
Manual	76	76	100%	96.1%
VP 2000	38	38	100%	92.4%

Analytical Specificity

Refer to the Breast Cancer **PERFORMANCE CHARACTERISTICS** section of this package insert for analytical specificity results.

Reproducibility

The reproducibility of the PathVysion Kit II assay was evaluated using FFPE gastric cancer specimens. Four gastric cancer specimens (2 HER-2/*neu*-positive and 2 HER-2/*neu*-negative, as determined by an immunohistochemistry [IHC] assay) were tested in replicates of 4, over 5 nonconsecutive days (1 run per day). Each slide was evaluated for quality and enumerated by 3 readers to determine HER-2/*neu* amplification status. The testing results of HER-2/*neu* amplification status were compared to the results of the IHC assay to evaluate reproducibility of the PathVysion Kit II assay. The PathVysion Kit II assay demonstrated reproducibility with 100% overall agreement for both reader-to-reader and day-to-day (run-to-run) analyses. Results are shown in **Table 26** and **Table 27**.

Table 26. Reproducibility: Reader-to-Reader

Reader	No. in Agreement with IHC	No. in Disagreement with IHC
1	80	0
2	80	0
3	80	0

Percent agreement: (240/240) = 100%

Table 27. Reproducibility: Day-to-Day (Run-to-Run)

Day/Run	No. in Agreement with IHC	No. in Disagreement with IHC
1	48	0
2	48	0
3	48	0
4	48	0
5	48	0

Percent agreement: (240/240) = 100%

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TECHNICAL ASSISTANCE:

For technical assistance, call Abbott Molecular Technical Services at +49-6122-580, email molecularsupport@abbott.com, or visit the Abbott Molecular Web site at <http://www.abbottmolecular.com>.

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