PATHWAY® HER 2 (clone CB11)

Catalog number 760-2694: 50 Test

Caution: Federal law restricts this device to sale by or on the order of a physician, or to a clinical laboratory; and its use is restricted to, by, or on the order of a physician.

I. INDICATIONS AND USE

A. Intended Use

This antibody is intended for *in vitro* diagnostic use.

Ventana Medical Systems, Inc.'s (Ventana) PATHWAY HER 2 (clone CB11) is a mouse monoclonal antibody intended for laboratory use for the semi-quantitative detection of c-erbB-2 antigen in sections of formalin-fixed, paraffin-embedded normal and neoplastic tissue on a Ventana automated immunohistochemistry slide staining device. It is indicated as an aid in the assessment of breast cancer patients for whom Herceptin® treatment is considered.

Note: All of the patients in the Herceptin clinical trials were selected using a clinical trial assay. None of the patients in those trials were selected using PATHWAY HER 2. The PATHWAY HER 2 was compared to the DAKO HercepTest® on an independent sample and found to provide acceptably concordant results. The actual correlation of PATHWAY HER 2 to clinical outcome has not been established.

B. Summary and Explanation

PATHWAY HER 2 is a mouse monoclonal antibody (clone CB11) directed against the internal domain of the c-erbB-2 oncoprotein. c-erbB-2 oncoprotein was cloned and characterized by Akiyama et al in 1986.[1] It is an approximately 185 kD transmembrane glycoprotein which is structurally similar to epidermal growth factor receptor (EGFR). The protein is associated with tyrosine kinase activity similar to that of several growth factor receptors, and to that of the transforming proteins of the *src* family. The coding sequence is consistent with an extracellular binding domain and an intracellular kinase domain. This suggests that c-erbB-2 may be involved in signal transduction when bound by a growth factor, stimulating mitogenic activity.[1]

Clone CB11 has been shown to react with a 190 kD protein from SK-BR-3 cell lysates via Western blotting.[7] SK-BR-3 is a breast carcinoma cell line which has a 128-fold over expression of c-erbB-2 mRNA.[7] The size of the band identified correlates well with that reported by Akiyama et al for c-erbB-2 (185 kD).[1]

Immunohistochemistry has been used to detect specific antigens in cells or tissue since 1950.[6] The use of enzymes and peroxidase as markers for immunohistochemistry was reported by Nakane and Pierce in 1967.[18] The increased sensitivity of the avidin-biotin-peroxidase detection system over the enzyme labeled antibody method was documented by Hsu et al in 1981.[14]

The c-erbB-2 oncoprotein is expressed at a level detectable by immunohistochemistry in up to 20 percent of adenocarcinomas from various sites. Between 15 and 30 percent of invasive ductal cancers are positive for c-erbB-2.[9] Almost all cases of Paget's disease of breast[15] and up to 90 percent of cases of ductal carcinoma *in situ* of comedo type are positive.[9] The immunohistochemical detection of c-erbB-2 protein overexpression is also used as an aid in determination of patients for whom Herceptin therapy is indicated.[12]

Staining results in normal tissues, neoplastic tissues, and 651 cases of breast carcinoma with PATHWAY HER 2 were evaluated by Ventana. In the 78 normal tissues tested, expression was consistent with the published literature in that the majority was cytoplasmic. Unexpected staining patterns occurred with one case of kidney and one case of tonsil showing cell membrane staining and one case of thyroid exhibiting extracellular staining. Of the 19 neoplastic tissues tested, cytoplasmic staining was seen in cancer cells of the lung, prostate, colon, bladder, cervix and ovary. 151 breast carcinomas were evaluated with Ventana PATHWAY HER 2 in relation to estrogen receptor expression. Strong c-erbB-2 positivity in these cases exhibited an inverse correlation with estrogen receptor which is consistent with published literature.[2, 11, 22, 26] Intense c-erbB-2 staining of the cell membrane was within the range of positivity reported in the literature for intense membranous staining - 10.6 % (16/151) vs. 9 to 16.5 %.[7]

450 additional breast carcinoma cases were studied to evaluate the correlation of staining with that of HercepTest, another immunohistochemical test for aiding in the selection of patients for treatment with Herceptin. There is a significant correlation of staining between these two tests. See section E, Summary of Expected Results, for further information.

Ventana PATHWAY HER 2, in combination with Ventana Detection Kits, utilizes biotinylated secondary antibodies to locate the bound PATHWAY HER 2 primary antibody (produced by using a synthetic peptide corresponding to a site on the internal domain of the c-erbB-2 oncoprotein). This is followed by the binding of an avidin/streptavidin-enzyme conjugate to the biotin. The complex is then visualized using a precipitating enzyme generated product.

The use of Ventana pre-diluted PATHWAY HER 2 (clone CB11) and ready-to-use detection kits, in combination with a Ventana automated slide stainer, reduces the possibility of human error and inherent variability resulting from individual reagent dilution, manual pipetting, and manual reagent application.

C. Clinical Significance

Breast cancer is the most common carcinoma occurring in women, and the second leading cause of cancer related death. In North America, a woman's chance of contracting breast cancer is one in eight.[23] Early detection and appropriate treatment therapies can significantly affect overall survival.[3] Small tissue samples may be easily used in routine immunohistochemistry (IHC), making this technique, in combination with antibodies that detect antigens important for carcinoma interpretation, an effective tool for the pathologist in his or her diagnosis and prognosis of disease. One important marker in breast cancer today is c-erbB-2 oncoprotein (c-erbB-2).

c-erbB-2 is an intracellular membrane protein detected in the cellular membrane.[7] It is closely related to EGFR and, like EGFR, has tyrosine kinase activity.[2] Gene amplification and the corresponding overexpression of c-erbB-2 has been found in a variety of tumors, including breast carcinomas.[7,19]

The newly cleared therapeutic Herceptin has been shown to benefit some breast carcinoma patients by arresting, and in some cases reversing the growth of their cancer.[12] The drug is a humanized monoclonal antibody that binds to HER-2/*neu* protein on cancer cells. Thus only patients with HER-2/*neu* positive breast carcinomas should benefit from treatment with Herceptin. *In vitro* diagnostics for the determination of HER-2/*neu* status in breast carcinomas are important to aid the clinician in determination of therapy with Herceptin.

Interpretation of the results of any detection system for c-erbB-2 must take into consideration the fact that c-erbB-2 is expressed in both breast cancer tumors and healthy tissue, albeit at differing levels and with different patterns of expression.[8] Histological tissue preparations have the advantage of intact tissue morphology to aid in the interpretation of the c-erbB-2 positivity of the sample. All histological tests should be interpreted by a specialist in breast cancer morphology, and/or pathology, and the results should be used in conjunction with other clinical and laboratory data.

D. Principles and Procedures

Ventana PATHWAY HER 2 (clone CB11) is a mouse monoclonal antibody, which binds to c-erbB-2 in paraffin-embedded tissue sections. The specific antibody is localized by a biotin-conjugated secondary antibody formulation that recognizes rabbit and mouse immunoglobulins. This step is followed by the addition of an avidin/streptavidin-enzyme conjugate that binds to the biotin present on the secondary antibody. The specific antibody-secondary antibody-avidin/streptavidin-enzyme complex is then visualized with a precipitating enzyme reaction product. Each step is incubated for a precise time and temperature. At the end of each incubation step, the Ventana automated slide stainer washes the sections to stop the reaction and remove unbound material that would hinder the desired reaction in subsequent steps. It also applies Liquid Coverslip™, which minimizes evaporation of the aqueous reagents from the specimen-containing slide. Results are interpreted using a light microscope and aid in the differential diagnosis of pathophysiological processes, which may or may not be associated with a particular antigen.

For further information refer to the Ventana automated slide stainer Operator's Manual.

II. MATERIALS AND METHODS

A. Reagents Provided

PATHWAY HER 2 consists of one dispenser of c-erbB-2 primary antibody (clone CB11) and contains approximately 5 ml (50 test) of pre-diluted reagent. The dispenser contains approximately 3 μg (50 test) of mouse monoclonal antibody (clone CB11) directed against the internal domain of the c-erbB-2 oncoprotein. c-erbB-2 oncoprotein was cloned and characterized by Akiyama et al in 1986.[1] It is an approximately 185 kD transmembrane glycoprotein which is structurally similar to epidermal growth factor receptor (EGFR). Clone CB11 has been shown to react with a 190 kD protein from SK-BR-3 cell lysates (a breast carcinoma cell line which has a 128-fold over expression of c-erbB-2 mRNA[7]) via Western blotting.[7] The size of the band identified correlates well with that reported by Akiyama et al for the size of the purified protein.[1] Two additional bands at 150 kD and 130 kD are also detected on heavily loaded gels. It has been suggested that the 150 kD and 130 kD bands visualized correspond to precursor or cytoplasmic versions of c-erbB-2.[7, 8]

The immunogen used to develop c-erbB-2 primary antibody, clone CB11 was a 17 amino acid synthetic peptide corresponding to the internal domain of the protein near the C-terminus.[7] The region of the c-erbB-2 protein was selected on the basis of computer-predicted antigenicity. The antibody is produced from hybridoma culture supernatants with no further purification. The antibody is diluted in 0.1 M phosphate buffered saline with 0.3 % carrier protein and 0.05 % ProClin 300, a preservative containing the active ingredients 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one (Supelco Catalog Number 4-8126). There is trace fetal calf serum, approximately 0.25 %, present from the stock solution.

Total protein concentration of the reagent is approximately 2.25 mg/ml. Specific antibody concentration is approximately 0.63 μg/ml (0.0003 % of the total protein). Clone CB11 is immunoglobulin class IgG_1, light chain kappa. There is no known irrelevant antibody in the preparation. The specificity of the antibody was shown by immunoprecipitation to have molecular mass of 190 kD.[7]

B. Reconstitution, Mixing, Dilution, Titration

Ventana PATHWAY HER 2 is optimized for use on a Ventana automated slide stainer in combination with Ventana detection kits. No reconstitution, mixing, dilution, or titration is required. Further dilution may result in loss of antigen staining. Any such change must be validated by the user. Differences in tissue processing and technical procedure in the user's laboratory may produce significant variability of results, necessitating regular performance of "in-house" controls (see section IV. B. Quality Control).

C. Materials and Reagents Needed But Not Provided

The following reagents and materials may be required for staining but not provided with Ventana PATHWAY HER 2:

1. 10 % neutral buffered formalin (Baxter Cat. No. C4320-101 or equivalent, or refer to Theory and Practice of Histotechnology by Sheehan and Hrapchak[24])
2. Negative tissue control slide (normal breast tissue)
3. Positive tissue control slide (breast carcinoma tissue)
4. Ventana PATHWAY HER 2 Control Slides (Cat. No. S9100)
5. Microtome
6. Microscope slides [Baxter Cat. No. M6164-plus (silanized), or Baxter Polysine Cat. No. M6143, or polylysine-coated, or equivalent]
7. Drying oven capable of maintaining a temperature of 70 °C ± 5 °C
8. Ventana bar code labels (Ventana Cat. No. 451-000, 451-800, or 451-801 for negative control and any one of Cat. No. 451-001 through 451-175 for PATHWAY HER 2) (appropriate for negative control and primary antibody being tested)
9. Xylene (histological grade)
10. Ethanol or reagent alcohol (histological grade)
    -100 % solution: Undiluted ethanol or reagent alcohol
    -95 % solution: Mix 95 parts of ethanol or reagent alcohol with 5 parts of deionized water
    -80 % solution: Mix 80 parts of ethanol or reagent alcohol with 20 parts of deionized water
11. Deionized/distilled water
12. Ventana ES®, NexES® IHC, or BenchMark® Series (BenchMark, BenchMark XT, and BenchMark LT) automated slide stainer
13. Ventana Basic DAB, AEC, or Alkaline Phosphatase Red detection kit
14. Detection kit specific software (ES automated slide stainer only)
15. Ventana APK Wash (10X) (ES and NexES IHC automated slide stainers)
16. Ventana Reaction Buffer (10X) (BenchMark Series automated slide stainers)
17. Ventana EZ Prep™ (10X) (BenchMark Series automated slide stainers)
18. Ventana Liquid Coverslip™ (Low Temperature) (ES and NexES IHC automated slide stainers)
19. Ventana Liquid Coverslip (High Temperature) (BenchMark Series automated slide stainers)
20. Ventana Cell Conditioning 2 (Pre-dilute) (BenchMark Series automated slide stainers)
21. Ventana Endogenous Biotin Blocking Kit
22. Ventana Antibody Diluent
23. Ventana Negative Control Mouse Ig, which contains MOPC 21, a mouse myeloma protein of subclass IgG1, kappa light chain. Negative Control Mouse Ig is nonreactive with human antigens. An equivalent negative control may be used.
24. Mounting Medium: Pro-Texx Mounting Medium (Scientific Products Cat. No. M7635-5) or equivalent for use with DAB Detection Kit or Immu-Mount (Shandon Cat. No. 99900402) or equivalent for use with AEC Detection Kit.
25. Cover Glass (Baxter Scientific Products Cat. Nos. M6045-7, M6045-8, M6045-9, M6045-10, or equivalent, depending on size of tissue)
26. Light microscope (20-80X)
27. Staining jars or baths
28. Timer (capable of 3-10 minute intervals)
29. Wash bottles
30. Absorbent wipes
31. Hydrogen peroxide
32. Ventana Hematoxylin counterstain
33. Ventana Bluing Reagent

D. Storage and Handling

Store PATHWAY HER 2 at 2 °C to 8 °C. Do not freeze.

Replace the cap and store dispenser in an upright position when not in use on the instrument. This will insure proper reagent delivery. Use care to avoid damaging dispensers.

Ventana PATHWAY HER 2 should be allowed to stand at least 30 minutes at room temperature prior to use. PATHWAY HER 2 must be returned to storage conditions identified above immediately after use. Any storage conditions other than those specified in the package insert must be validated by the user.

Every Ventana PATHWAY HER 2 dispenser is expiration dated. Do not use reagent beyond expiration date for prescribed storage method. The product has been designed to have 12 months dating after the date of manufacture.

E. Indications of Instability

When properly stored, the reagent should be stable to the dating indicated on the label. Ventana has designed the PATHWAY HER 2 to have one year stability from the date of manufacture. The user must honor the expiration date on the label. There are no obvious signs to indicate instability of this product. Therefore, positive and negative controls should be run simultaneously with unknown specimens. Positive controls assure that the specimen staining was carried out correctly. Negative reagent controls are used to assess non-specific staining which must be taken into consideration when interpreting results. Whenever positive control material shows a decrease in staining, it is a possible indication of reagent instability, and Ventana Technical Consultation Center (800-227-2155) should be contacted immediately.

F. Specimen Collection and Preparation for Analysis

Formalin-fixed, paraffin-embedded tissues which have been antigen-enhanced are suitable for use with Ventana PATHWAY HER 2 when used with Ventana detection kits and a Ventana automated slide stainer (See Section II. C. Materials and Reagents Needed, But Not Provided).
The recommended fixative is 10 % neutral buffered formalin. The amount used is 15 to 20 times the volume of tissue. No fixative will penetrate more than 2 to 3 mm of solid tissue or 5 mm of porous tissue in a 24 hour period. A 3 mm or smaller section of tissue should be fixed no less than 4 hours and no more than 8 hours. Fixation can be performed at room temperature (15-25 °C).[24]
Osseous tissues should be decalcified prior to tissue processing to facilitate tissue cutting and prevent damage to microtome blades.[24]

Properly fixed and embedded tissues expressing the antigen will keep 2 years if stored in a cool place (15-25 °C). The Clinical Laboratory Improvement Act (CLIA) of 1988 requires in 42 CFR 493.1259(b) that "The laboratory must retain stained slides at least ten years from the date of examination and retain specimen blocks at least two years from the date of examination".[4]

Approximately 5 μm thick sections should be cut and picked up on glass slides. The slides should either be silanized or coated with a polylysine compound. Tissue should be dried by placing the slides in a 70 °C (+/- 5°C) oven for at least two hours, but not longer than 24 hours.[24] Studies at Ventana indicate unbaked cut tissue and cell line sections affixed to glass slides and stored at room temperature are stable for 9 months. Each laboratory should validate the cut slide stability for there own procedures and environmental storage conditions.

Deparaffinize by immersion in two xylene baths for five minutes ± 1 minute each. Rehydrate and bring to water with 3 minutes ± 1 minute each in two baths containing 100 % ethanol, 3 minutes ± 1 minute in 95 % ethanol, 3 minutes ± 1 minute in 80 % ethanol, and 10 dips in water.[24]

Use an appropriate antigen enhancement procedure.**Error! Reference source not found.**[25]

Blot-dry frosted end of processed tissue slides, ensuring that the tissue sections do not dry. Properly label processed slides with bar codes and place in Wash Solution until ready to load on Ventana automated slide stainer.

It is recommended that slides be loaded on the instrument within an hour, if at all possible. They may be left in wash solution for up to 2 hours if necessary, as long as tissue is not allowed to dry.

PATHWAY HER 2 works well with antigen enhanced, formalin-fixed, paraffin-embedded tissues. Variable results may occur as a result of special processes such as decalcification of bone marrow preparations.

III. Safety Issues

A. Precautions

1. This antibody is intended for *in vitro* diagnostic use.
2. Take reasonable precautions when handling reagents. Use disposable gloves when handling suspected carcinogens or toxic materials, for example xylene or formaldehyde.
3. Do not smoke, eat or drink in areas where specimens or reagents are being handled.
4. Avoid contact of eyes and mucous membranes with reagents. If reagents come in contact with sensitive areas, wash with copious amounts of water.
5. Patient specimens and all materials coming into contact with them should be handled as if capable of transmitting infection and disposed of with proper precautions. Never pipette by mouth and avoid contact of reagents and specimens with skin and mucous membranes.
6. Avoid microbial contamination of reagents as this could produce incorrect results.
7. Incubation times and temperatures other than those specified may give erroneous results. Any such change must be validated by the user.
8. The reagents have been optimally diluted and further dilution may result in loss of antigen staining. Any such change must be validated by the user.
9. Ventana recommended protocols are described in section IV. A. Step by Step Procedure. Deviation from these protocols may produce erroneous results. All protocols must be validated by the user. Users who deviate from recommended test procedures must accept responsibility for interpretation of patient results under these circumstances.

B. Toxicity of Antibody Solution

Symptoms of overexposure to ProClin 300, the preservative in the antibody reagent, may include skin and eye irritation and irritation to mucous membranes and upper respiratory tract. The concentration of ProClin 300 in this product is 0.05 %. This antibody solution does not meet the OSHA criteria for a hazardous substance. Refer to the Material Safety Data Sheet enclosed with each shipment of product, or call (800) 227-2155 for a copy.

IV. Instructions for Use

A. Step by Step Procedure

Ventana PATHWAY HER 2 was developed for use on a Ventana automated slide stainer in combination with Ventana detection kits and accessories.

ES and NexES IHC automated slide stainers

Recommended staining protocol for the Ventana ES and NexES IHC automated slide stainers are listed in Table 1 below. These parameters can be displayed, printed and edited according to the procedure in the Ventana automated slide stainer Operator's Manual. Other operating parameters for the automated slide stainers have been preset at the factory.

Table 1. Recommended Protocols for PATHWAY HER 2 on ES and NexES IHC automated slide stainers

PROCEDURE TYPE	ENZYME DIGEST	PRIMARY INCUBATION	COUNTERSTAIN
PARAFFIN	NONE	32 MIN	HEMATOXYLIN

The procedure for staining c-erbB-2 with PATHWAY HER 2 on the Ventana ES and NexES IHC automated slide stainers is as follows (Refer to the Operator's Manual for specific details on the operation of the Ventana automated slide stainer).

1. The PATHWAY HER 2 dispenser, appropriate detection kit dispensers, and desired accessory reagents are loaded onto the reagent tray and placed on the Ventana automated slide stainer.
2. Each slide must be labeled with the appropriate bar code specifying the staining procedure and PATHWAY HER 2. The slide bar codes should be applied after the antigen enhancement procedure.
3. Load the deparaffinized/antigen-enhanced sections from 1X APK Wash Solution pH 7.6, being careful not to allow the tissue to dry.

Ventana PATHWAY HER 2 has been optimized to work in combination with Ventana detection kits to provide the greatest specific staining-to-background ratio. The following is the sequence of events carried out by the Ventana ES and NexES IHC automated slide stainers.

1. Inhibitor solution is applied to decrease the endogenous peroxidase activity if a peroxidase-based detection system is used. The inhibitor on the slide is incubated with mixing for 4 minutes at 37 °C.
2. The slide is rinsed; the optimized PATHWAY HER 2 is applied and incubated with mixing for 4 to 32 minutes at 37 °C (see Specific Limitation #3).
3. The slide is rinsed; biotinylated secondary antibody is applied and incubated with mixing for 8 minutes at 37 °C.
4. The slide is rinsed; avidin/streptavidin-enzyme conjugate is applied and incubated with mixing for 8 minutes at 37 °C.
5. The slide is rinsed; chromogenic enzyme substrate is added and incubated (the time is indicated in each Detection Kit insert) with mixing at 37 °C.
6. The slide is rinsed; if a DAB Detection Kit is used, copper DAB enhancer is applied with mixing for 4 minutes at 37 °C.
7. Counterstain (selectable): if counterstain and/or post counterstain is selected, each will be applied to the slide and incubated with mixing for the selected time interval at 37 °C. Ventana Hematoxylin counterstain and Bluing Reagent post counterstain are recommended for this protocol, with 4 minute incubation for each.

BenchMark automated slide stainer

Recommended staining protocol selected in the procedure menu for the Ventana BenchMark automated slide stainer is *BMK PATHWAY HER 2*. These parameters can be displayed, printed and edited according to the procedure in the Ventana automated slide stainer Operator's Manual. Other operating parameters for the automated slide stainers have been preset at the factory.

The procedure for staining c-erbB-2 with PATHWAY HER 2 on the Ventana BenchMark automated slide stainer is as follows (Refer to the Operator's Manual for specific details on the operation of the Ventana automated slide stainer).

1. The PATHWAY HER 2 dispenser, appropriate detection kit dispensers, and desired accessory reagents are loaded onto the reagent tray and placed on the Ventana BenchMark automated slide stainer.
2. Each slide must be labeled with the appropriate bar code specifying the staining procedure and PATHWAY HER 2.
3. Load the labeled slides onto the slide carousel.

Ventana PATHWAY HER 2 has been optimized to work in combination with Ventana detection kits to provide the greatest specific staining-to-background ratio. The following is the sequence of events carried out by the Ventana BenchMark automated slide stainer.

1. Deparaffinization (selectable): EZ Prep is applied and incubated for 12 minutes at 76 °C. On-instrument deparaffinization is recommended.
2. Cell conditioning: The slide is rinsed; Cell Conditioning 2 (pre-dilute) is applied and incubated with mixing for 32 minutes at 95 °C.
3. The slide is rinsed; Inhibitor solution is applied to decrease the endogenous peroxidase activity. The inhibitor on the slide is incubated with mixing for 4 minutes at 42 °C.
4. The slide is rinsed; the optimized PATHWAY HER 2 is applied and incubated with mixing for 32 minutes at 42 °C (see Specific Limitation #3).
5. A/B Block (selectable): The slide is rinsed; Blocker A is applied and incubated with mixing for 4 minutes. The slide is rinsed; Blocker B is applied and incubated with mixing for 4 minutes. This step utilizes the Ventana Endogenous Biotin Blocking Kit; blocking is recommended for this assay to reduce background staining due to endogenous biotin. Rinse step prior to Biotinylated Ig secondary antibody dispense is omitted if this step is selected.
6. The slide is rinsed; Biotinylated Ig secondary antibody is applied and incubated with mixing for 8 minutes at 42 °C.
7. The slide is rinsed; avidin-HRPO enzyme conjugate is applied and incubated with mixing for 8 minutes at 42 °C.
8. The slide is rinsed; DAB and DAB H_2O_2 are added and incubated together for 8 minutes with mixing at 42 °C.
9. The slide is rinsed; copper DAB enhancer is applied with mixing for 4 minutes at 42 °C.
10. Counterstain (selectable): if counterstain and/or post counterstain is selected, each will be applied to the slide and incubated with mixing for the selected time interval at 42 °C.

BenchMark XT and BenchMark LT automated slide stainers

Recommended staining protocol selected in the procedure menu for the Ventana BenchMark XT and BenchMark LT automated slide stainers is *XT PATHWAY HER-2 V.1*. These parameters can be displayed, printed and edited according to the procedure in the Ventana automated slide stainer Operator's Manual. Other operating parameters for the automated slide stainers have been preset at the factory.

The procedure for staining c-erbB-2 with PATHWAY HER 2 on the Ventana BenchMark XT and BenchMark LT automated slide stainer is as follows (Refer to the Operator's Manual for specific details on the operation of the Ventana automated slide stainer).

1. The PATHWAY HER 2 dispenser, appropriate detection kit dispensers, and desired accessory reagents are loaded onto the reagent tray and placed on the Ventana BenchMark automated slide stainer.
2. Each slide must be labeled with the appropriate bar code specifying the staining procedure and PATHWAY HER 2.
3. Load the labeled slides onto the slide carousel.

Ventana PATHWAY HER 2 has been optimized to work in combination with Ventana detection kits to provide the greatest specific staining-to-background ratio. The following is the sequence of events carried out by the Ventana BenchMark XT and BenchMark LT automated slide stainer.

1. Deparaffinization (selectable): EZ Prep is applied and incubated for 12 minutes at 77 °C. On-instrument deparaffinization is recommended.
2. Cell conditioning: The slide is rinsed; Cell Conditioning 2 (pre-dilute) is applied and incubated with mixing for 36 minutes at 95 °C.
3. The slide is rinsed; Inhibitor solution is applied to decrease the endogenous peroxidase activity. The inhibitor on the slide is incubated with mixing for 4 minutes at 37 °C.
4. NeuVision Blocking Reagent (selectable): do not select.
5. The slide is rinsed; the optimized PATHWAY HER 2 is applied and incubated with mixing for 32 minutes at 37 °C (see Specific Limitation #3).
6. A/B Block (selectable): The slide is rinsed; Blocker A is applied and incubated with mixing for 4 minutes. The slide is rinsed; Blocker B is applied and incubated with mixing for 4 minutes. This step utilizes the Ventana Endogenous Biotin Blocking Kit; blocking is recommended for this assay to reduce background staining due to endogenous biotin. Rinse step prior to Biotinylated Ig secondary antibody dispense is omitted if this step is selected.
7. The slide is rinsed; Biotinylated Ig secondary antibody is applied and incubated with mixing for 8 minutes at 37 °C.
8. The slide is rinsed; avidin-HRPO enzyme conjugate is applied and incubated with mixing for 8 minutes at 37 °C.
9. The slide is rinsed; DAB and DAB H_2O_2 are added and incubated together for 8 minutes with mixing at 37 °C.
10. The slide is rinsed; copper DAB enhancer is applied with mixing for 4 minutes at 37 °C.
11. Counterstain (selectable): if counterstain and/or post counterstain is selected, each will be applied to the slide and incubated with mixing for the selected time interval at 37 °C. Ventana Hematoxylin counterstain and Bluing Reagent post counterstain are recommended for this protocol, with 4 minute incubation for each.
12. NeuVision Counterstain (selectable): do not select.

Post-staining Procedure

Slides may be removed from the instrument, rinsed in detergent solution, dehydrated, cleared, (do not dehydrate or clear for AEC chromogen) and coverslipped using a mounting medium described in Section II C item 24 and cover glass described in Section II C item 25. The stained slides should be read within two to three days of staining and should be stable for up to two weeks if properly stored at room temperature (15-25 °C).

B. Quality Control Procedures

Differences in tissue processing and technical procedures in the user's laboratory may produce significant variability in results, necessitating regular performance of in house controls in addition to the following procedures. Consult the quality control guidelines of "Special report: Quality control in Immunohistochemistry"[10] and/or the Proposed NCCLS guideline for IHC.[20]

1. Cell Line Controls

   Ventana has available as a separate product formalin-fixed cell line controls embedded in paraffin, sectioned and placed on charged slides (catalog # S9100). Cell line controls may be useful for a preliminary validation of the processing method used for staining slides with PATHWAY HER 2. Ventana has available three cell line controls characterized by *in situ* hybridization for gene copy number and by Scatchard analysis for receptor content. When processed and stained appropriately, the cell lines should stain as described in Table 2. If the indicated staining, especially the 1+ staining in greater than 10% of the cells, is not evident in the Level 2 cell line control, the staining of the tissues should be repeated.

Table 2. Characterization of c-erbB-2 in Cell Line Controls

Control Slide:	Cell Line:	Gene Copy # (Avg.)	Receptor #	IHC Staining Intensity	% Cells Staining
Level 1	MDA-MB-468	2.5	8.45E+04	0	100%
Level 2	T-47D	4.8	1.13E+05	1+	>10%
Level 3	SK-BR-3	17	2.02E+05	3+	100%

2. Positive Tissue Control

   A positive control tissue fixed and processed in the same manner as the patient specimens must be run for each set of test conditions and with every PATHWAY HER 2 staining procedure performed by the instrument. This tissue should contain both positive staining cell/tissue components and negative cell/tissue components and serve as both the positive and negative control tissue. Control tissues should be fresh autopsy/biopsy/surgical specimens prepared and fixed as soon as possible in a manner identical to test sections. Such tissues may monitor all steps of the analysis, from tissue preparation through staining. Use of a tissue section fixed or processed differently from the test specimen provides control for all reagents and method steps except fixation and tissue processing.

A tissue with weak positive staining is more suitable than strong positive staining for optimal quality control and to detect minor levels of reagent degradation. Ideally, a tissue which is known to have weak, but positive staining should be chosen to ensure that the system is sensitive to small amounts of reagent degradation or problems with the IHC methodology. Generally, however, neoplastic tissue that is positive for c-erbB-2 is strongly positive due to the nature of the pathology (overexpression). An example of tissue to use as a positive control with PATHWAY HER 2 is intraductal adenocarcinoma of the breast demonstrating positivity for c-erbB-2. The positive staining cells/tissue components (cell membrane staining of ductal cells) are used to confirm that PATHWAY HER 2 was applied and the instrument functioned properly.

Known positive tissue controls should only be utilized for monitoring the correct performance of processed tissues and test reagents, NOT as an aid in formulating a specific diagnosis of patient samples. If the positive tissue controls fail to demonstrate positive staining, results with the test specimens should be considered invalid.

3. Negative Tissue Control

Use a tissue control known to be fixed, processed and embedded in a manner identical to the patient sample(s) with each staining run to verify the specificity of PATHWAY HER 2 for demonstration of c-erbB-2, and to provide an indication of specific background staining (false positive staining). Also, the variety of different cell types in most tissue sections can be used by the laboratorian as internal negative control sites to verify PATHWAY HER 2 performance specifications. For example, the same tissue used for the positive tissue control (intraductal adenocarcinoma of the breast) may be used as the negative tissue control. The non-staining components (surrounding stroma, lymphoid cells and blood vessels) should demonstrate absence of specific staining, and provide an indication of specific background staining. Alternatively, normal breast tissue is an adequate negative control tissue. If specific staining occurs in the negative tissue control sites, results with the patient specimens should be considered invalid.

4. Nonspecific Negative Reagent Control

A negative reagent control must be run from every tissue block stained on the Ventana automated slide stainer to aid in the interpretation of each patient result. A negative reagent control is used in place of the primary antibody to evaluate nonspecific staining and allow better interpretation of specific staining at the antigen site. This provides an indication of nonspecific background staining for each slide. In place of the primary antibody, stain the slide with Ventana CONFIRM Negative Control Mouse Ig, a mouse myeloma protein (IgG_1, kappa) directed against an antigen not found in human specimens, or with an appropriate substitute that has been produced in the same way as the primary antibody and in the same matrix solution. The mouse myeloma protein is the ideal negative control because it is nonspecific, produced in the same way as the primary antibody and of murine origin of the same isotype. If an alternative negative reagent control is used, dilute to the same dilution as the primary antibody/antiserum in Ventana Antibody Diluent. Approximately 0.25 % fetal calf serum is retained in PATHWAY HER 2. Addition of 0.25 % fetal calf serum in Ventana Antibody Diluent is also suitable for use as a nonspecific negative reagent control. Diluent alone may be used as a less desirable alternative to the previously described negative reagent controls. The incubation period for the negative reagent control should correspond to that of the primary antibody.

When panels of several antibodies are used on serial sections, the negatively staining areas of one slide may serve as a negative/non-specific binding background control for other antibodies.

To differentiate endogenous enzyme activity or nonspecific binding of enzymes from specific immunoreactivity, additional patient tissues may be stained exclusively with substrate-chromogen or enzyme complexes (avidin-biotin, streptavidin) and substrate-chromogen, respectively.

5. Unexplained Discrepancies

Unexplained discrepancies in control results should be referred to Ventana Technical Consultation Center (800-227-2155) immediately. If quality control results do not meet specifications, patient results are invalid. See Troubleshooting, section of this insert. Identify and correct the problem, then repeat the patient samples

6. Assay Verification

Prior to initial use of this antibody in the user's laboratory or if there is a change of lot number, the specificity of the antibody should be verified by staining a number of positive and negative tissues with known performance characteristics. Refer to the quality control procedures previously outlined in this section of the product insert and to the quality control recommendations of the CAP certification program for immunohistochemistry[5] and/or the NCCLS IHC guideline.[20] These quality control tests should be repeated for each new lot or whenever there is a change of lot number of one of the reagents in a matched set or a change in assay parameters. Quality control cannot be meaningfully performed on an individual reagent in isolation since the matched reagents, along with a defined assay protocol, must be tested in unison before using a kit for diagnostic purposes. Tissues listed in the Summary of Expected Results are suitable for assay verification.

Assay verification on a daily basis may be accomplished through the proper use of the above mentioned positive and negative controls, as described in this section (see Table 3 below). In addition, it is recommended that on a monthly basis, the c-erbB-2 positive tissue control be stained and compared to the same tissue control stained the previous month. Comparison of controls stained at monthly intervals serves to monitor the assay stability, sensitivity, specificity, and reproducibility.

All quality control requirements should be performed in conformance with local, state and/or federal regulations or accreditation requirements.

Table 3. The Purpose of Daily Quality Control

Tissue: Fixed and processed like patient sample	Specific antibody and secondary antibody	Nonspecific Antibody* or Ventana Antibody Diluent (#251-018) plus same secondary antibody as used with specific antibody
Positive control: tissue or cells containing target antigen to be detected (could be located in patient tissue). The ideal control is weakly positive staining tissue to be most sensitive to antibody degradation.	Controls all steps of the analysis. Validates reagent and procedures used for staining	Detection of non-specific background staining.
Negative Control: Tissue or cells to be negative (could be located in patient tissue or positive control tissue)	Detection of unintended antibody cross-reactivity to cells/cellular components	Detection of non-specific background staining
Patient Tissue	Detection of specific staining	Detection of non-specific background staining

*= Same source and type as the specific antibody but not directed against any human antigen. To detect non-specific antibody binding, e.g., binding of Fc portion of antibody by the tissue.

C. Interpretation of Staining

Scoring conventions for the interpretation of PATHWAY HER 2:

Breast carcinomas that are considered positive for c-erbB-2 must meet a threshold criteria for intensity of staining (2+ or greater on a scale of 0 to 3+) and percent positive tumor cells (greater than 10 %). Staining must also localize to the cellular membrane. Cytoplasmic staining may still be present, but this staining is not included in the determination of positivity. Three fields within the well-preserved and well-stained region of the tissue should be examined for intensity of staining and determination of completeness of the cytoplasmic membranous stain. Staining that completely encircles the cytoplasmic membrane should be scored as an intensity of "2+" or "3+". Partial staining of the membrane should be scored as a "1+". It may be necessary to examine borderline cases at 400X or higher magnification to discriminate between intensities of "1+" and "2+". In contrast to cases scored as an intensity of 3+, the staining scored as 2+ has a crisper and more clearly delineated ring, while cases scored as 3+ exhibit a very thick outline. Below is a quick reference chart for staining criteria. Refer to Ventana PATHWAY HER 2 Scoring Guide for a more detailed description with photographs of staining with PATHWAY HER 2.

Table 4. Criteria for Intensity of Cell Membrane Staining with PATHWAY HER 2

Staining Pattern	Score (Report to treating physician)	c-erbB-2 Staining Assessment
No membrane staining is observed	0	Negative
Faint, partial staining of the membrane	1+	Negative
Weak complete staining of the membrane, greater than 10% of cancer cells	2+	Positive
Intense complete staining of the membrane, greater than 10% of cancer cells	3+	Positive

Interpretation of staining within the context of controls:

The Ventana automated immunostaining procedure causes a colored reaction product to precipitate at the antigen sites localized PATHWAY HER 2 (Refer to the appropriate Ventana detection kit package insert for the expected color.) A qualified pathologist who is experienced in immunohistochemical procedures must evaluate positive and negative controls and qualify the stained product before interpreting results.

Positive Tissue Control: The positive tissue control stained with Ventana PATHWAY HER 2 should be examined first to ascertain that all reagents are functioning properly. The presence of rose red (3-amino-9-ethylcarbazole, AEC), bright pink (fast red) or reddish-brown (3,3'-diaminobenzidine tetrahydrochloride, DAB) reaction product with the target cells' membranes is indicative of positive reactivity. PATHWAY HER 2 shows more intense staining in ductal cells of breast carcinoma on the cell surface membrane, with occasional lesser cytoplasmic staining. It is imperative that only the intense membrane, and not cytoplasmic staining, be considered positive if a false-positive interpretation is to be avoided.[7] Staining in breast carcinoma is found only in a subset of cases (approximately 20 %). Morphologically, staining is found in ductal cells in a cell membrane pattern. Ductal cells sometimes react less intensely in a cytoplasmic pattern with the antibody. The surrounding stroma, lymphoid cells and blood vessels should be unreactive. If the positive tissue control fails to demonstrate positive staining, any results with the test specimens should be considered invalid.

The color of the reaction product may vary if substrate chromogens other than those stated are used. Refer to substrate package inserts for expected color reactions. Further, metachromasia may be observed in variations of the method of staining.[16]

Depending on the incubation length and potency of the hematoxylin used, counterstaining will result in a pale to dark blue coloration of the cell nuclei. Excessive or incomplete counterstaining may compromise proper interpretation of results.

Negative Tissue Control: The negative tissue control should be examined after the positive tissue control to verify the specific labeling of the target antigen by the primary antibody. The absence of specific staining in the negative tissue control confirms the lack of antibody cross-reactivity to cells/cellular components. The staining of normal breast is an adequate negative control tissue. Intact stromal and duct elements should show no intense staining in the membrane indicating that staining did not occur. If the tissue is counterstained, there may be staining around the outside of the cell, i.e. the interstitial spaces. If specific staining occurs in the negative tissue control, results with the patient specimen should be considered invalid.

Nonspecific staining, if present, will have a diffuse appearance. Sporadic light staining of connective tissue may also be observed in sections from excessively formalin-fixed tissues. Use intact cells for interpretation of staining results. Necrotic or degenerated cells will often stain nonspecifically.[17]

Patient Tissue: Patient specimens should be examined last. Positive staining intensity should be assessed within the context of any nonspecific background staining of the negative reagent control. Strong membrane staining is a much more reliable indicator of the presence of clinically significant c-erbB-2 than is cytoplasmic staining only.[7] The neoplastic cells of breast carcinomas show no reactivity in the majority of cases (approximately 80 %). Certain subsets of breast carcinomas exhibit higher frequencies of c-erbB-2 over-expression. Almost all cases of Paget's disease of breast[15] and up to 90 percent of cases of ductal carcinoma in situ of comedo type are positive.[9] c-erbB-2 may be detected among other neoplasms, particularly adenocarcinomas of the ovary, stomach, and bladder in frequencies similar to that of breast cancer (20 %).[9] As with any immunohistochemical test, a negative result means that the antigen in question was not detected, not that the antigen is absent in the cells/tissue assayed. If necessary use a panel of antibodies to aid in the identification of false negative reactions (see Section IV. B. Quality Control Procedures). The morphology of each tissue sample should also be examined utilizing a hematoxylin and eosin stained section when interpreting any immunohistochemical result. The patient's morphologic findings and pertinent clinical data must be interpreted by a qualified pathologist. Refer to Summary and Explanation, Limitations, and Summary of Expected Results for specific information regarding immunoreactivity.

D. Limitations

General Limitations:

1. Immunohistochemistry (IHC) is a multistep diagnostic process that consists of specialized training in the selection of the appropriate reagents; tissue selection, fixation, processing; preparation of the IHC slide; and interpretation of the staining results.
2. Tissue staining is dependent on the handling and processing of the tissue prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, sectioning or contamination with other tissues or fluids may produce artifacts, antibody trapping, or false negative results. Inconsistent results may be due to variations in fixation and embedding methods, or to inherent irregularities within the tissue.[17]
3. Excessive or incomplete counterstaining may compromise proper interpretation of results.
4. Unexpected negative staining in tumors may be due to loss of expression of antigen or loss of the gene(s) coding the antigen as a tumor dedifferentiates. Unexpected positive staining in tumors may be from expression of an antigen not usually expressed in normal cells or persistence or acquisition of an antigen in a dedifferentiated tumor that develops morphologic and immunohistochemical markers associated with another cell lineage. Histopathologic classification of tumors is not an exact science and some literature reports of unexpected staining are controversial.
5. The clinical interpretation of any positive or negative staining should be evaluated within the context of clinical presentation, morphology and other histopathological criteria. The clinical interpretation of any positive or negative staining should be complemented by morphological studies using proper positive and negative internal and external controls as well as other diagnostic tests. It is the responsibility of a qualified pathologist who is familiar with the proper use of IHC antibodies, reagents and methods, to interpret all of the steps used to prepare and interpret the final IHC preparation.
6. This product is not intended for use in flow cytometry. Performance characteristics have not been determined for flow cytometry.
7. Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit nonspecific staining with horseradish peroxidase.[21]
8. Reagents may demonstrate unexpected reactions in previously untested tissues. The possibility of unexpected reactions even in tested tissue groups cannot be completely eliminated due to biological variability of antigen expression in neoplasms, or other pathological tissues.[13] Contact Ventana Technical Consultation Center (800-227-2155) with documented unexpected reaction(s).
9. Normal/nonimmune sera from the same animal source as secondary antisera used in blocking steps may cause false negative or positive results due to auto-antibodies or natural antibodies.
10. False positive results may be seen due to non-immunological binding of proteins or substrate reaction products. They may also be caused by pseudoperoxidase activity (erythrocytes), endogenous peroxidase activity (cytochrome C), or endogenous biotin (e.g. liver, brain, breast, kidney) depending on the type of immunostain used.[17]

Specific Limitations:

1. Ventana PATHWAY HER 2 has been optimized for a 32 minute incubation time with antigen-enhanced tissue, in combination with Ventana detection kits and Ventana automated slide stainers. Due to variation in tissue processing, the time of incubation depends upon the degree of tissue fixation and/or the effectiveness of antigen enhancement and must be determined empirically. It may be necessary to increase or decrease PATHWAY HER 2 incubation time on individual specimens. Users who deviate from recommended test procedures must accept responsibility for interpretation of patient results under these circumstances.
2. Ventana PATHWAY HER 2, in combination with Ventana detection kits and accessories, demonstrates c-erbB-2 antigen that survives routine tissue fixation, processing and sectioning.

3. Primary antibody incubation time depends on the degree of tissue fixation and may range from 4 to 32 minutes. Ventana recommends 32 minutes for use with its detection kits. For further information about fixation variables refer to Immunomicroscopy: A Diagnostic Tool for the Surgical Pathologist.[25]
4. The following tissues have not been tested: parathyroid.
5. False negative cases may result from various factors, including true antigen decrease, loss or structural change during tumor "dedifferentiation" or terminal differentiation artifactual change during fixation or processing. As with any immunohistochemical test, a negative result means that the antigen was not detected, not that the antigen was absent in the cells/tissues assayed.
6. Neoplastic tissue is the recommended positive control tissue. While many normal human tissues react positively with PATHWAY HER 2, the staining pattern in normal tissues is generally cytoplasmic while neoplastic tissues, such as breast carcinoma show intense membranous staining.[7]
7. c-erbB-2 may be detected among other neoplasms, particularly adenocarcinomas of the ovary, stomach, and bladder in frequencies similar to that of breast cancer (20 %).[9]
8. Not all breast carcinomas are expected to be positive with PATHWAY HER 2. Only 9-16.5 % have been seen to be positive with Clone CB11.[7]
9. The degree of c-erbB-2 protein overexpression may be an important predictor of response to therapy with Herceptin. Data from clinical trials of Herceptin suggests that the beneficial treatment effects were primarily limited to patients whose tumor's overexpression of c-erbB-2 protein was 3+.[12]

E. Summary of Expected Results

1. Reproducibility

Intra-run reproducibility of staining with Ventana PATHWAY HER 2 was determined by staining 20 slides containing sections from the same neutral buffered formalin-fixed tissue. The procedure included pretreatment of the slides using antigen enhancement in an acidic buffer. Slides were then stained using a protocol consisting of 32 minute incubation of the primary antibody, Ventana's Basic DAB detection kit, and counterstaining with hematoxylin and bluing reagent. 20 of 20 slides stained positively. All slides stained with similar staining intensity. Users should verify within run reproducibility results by staining several sets of serial sections with low, medium, and high antigen density in a single run.

Inter-run reproducibility of staining with Ventana PATHWAY HER 2 was determined by staining slides containing sections from the same neutral buffered formalin-fixed tissue on 20 different runs. The procedure included pretreatment of the slides using antigen enhancement in an acidic buffer. Slides were then stained using a protocol consisting of 32 minute incubation of the primary antibody, Ventana's Basic DAB detection kit, and counterstaining with hematoxylin and bluing reagent. All slides stained with similar staining intensity. Users should verify between run reproducibility results by staining several sets of serial sections with low, medium, and high antigen density on different days.

Inter-laboratory staining reproducibility: Three laboratories, from separate institutions in the United States, participated in the inter-laboratory reproducibility study. Cut slides of four neutral buffered formalin-fixed breast carcinoma cases and the three neutral buffered formalin-fixed cell line controls were shipped to the sites for staining on a Ventana automated slide staining device. The slides were returned to Ventana and masked, then sent for evaluation by a qualified pathologist.

All three sites performed identical pretreatment procedures and staining protocols. The procedure included pretreatment of the slides using antigen enhancement in an acidic buffer. Slides were then stained using a protocol consisting of 32 minute incubation of the primary antibody, Ventana's Basic DAB detection kit, and counterstaining with hematoxylin and bluing reagent. All slides stained with similar staining intensity (varied by no more than 1 intensity level). No cases varied from clinically positive to clinically negative between the sites and no sites experienced invalid runs, based upon the performance of the controls. Controls included the three cell lines, a positive tissue control located on the same slide as the test case, and a second slide of each case stained with negative Ig reagent. Evaluating 3 inter-laboratory comparisons of the 2 cases staining positively, variation between 3+ intensity and 2+ intensity occurred four times out of a total of 22 staining events (18 %). Thus there can be some difficulty distinguishing a 2+ from a 3+ reaction.

Intra-laboratory, intra-technician and inter-technique staining reproducibility: The same three laboratories that participated in the inter-laboratory reproducibility study participated in an intra-laboratory (technician A vs. technician B) and intra-technician (technician A vs. technician A, 1 week apart) study, utilizing the same four breast carcinoma cases and cell line controls. The slides were returned to Ventana and masked, then sent for evaluation by a qualified pathologist. At all three sites intra- and inter-technician staining of all the slides stained with similar staining intensity (varied by no more than 1 intensity level). No cases varied from clinically positive to clinically negative between the sites. Evaluating 3 intra-laboratory comparisons (technician A run 1 vs. technician B) of the 2 cases staining positively, variation between 3+ intensity and 2+ intensity occurred one time out of a total of 16 staining events (6 %). Evaluating 3 intra-technician comparisons (technician A vs. technician A, 1 week apart) of the 2 cases staining positively, variation between 3+ intensity and 2+ intensity occurred three times out of a total of 14 staining events (21 %). Thus there can be some difficulty distinguishing a 2+ from a 3+ reaction.

Additionally these sites were invited to use their own in-house pretreatment methods with the study cases to evaluate inter-technique reproducibility. Again there were no differences of more than one intensity level between methodologies and no variation occurred that changed the clinical interpretation of the results. Evaluating 3 intra-technique comparisons (technician A Run 2 Ventana method vs. technician A Run 2 in-house method) of the 2 cases staining positively, variation between 3+ intensity and 2+ intensity occurred two times out of a total of 14 staining events (14 %). Thus staining with an in-house method was equivalent to the Ventana staining method.

Intra-investigator and inter-investigator scoring reproducibility: Five investigators participated in a scoring reproducibility study. Each investigator was sent the same set of twelve neutral buffered formalin-fixed breast carcinoma cases pre-stained with hematoxylin and eosin (H&E), PATHWAY HER 2, and negative Ig reagent. The procedure for preparing the slides used in this study included pretreatment of the slides using antigen enhancement in an acidic buffer. Slides were then stained using a protocol consisting of 32 minute incubation of the primary antibody (PATHWAY HER 2 or negative Ig reagent), Ventana's Basic DAB detection kit, and counterstaining with hematoxylin and bluing reagent. Investigators were provided with a scoring guide with photographs of breast carcinoma cases stained with PATHWAY HER 2, and the study protocol with directions for scoring. Investigators were also instructed to call Ventana if they had questions. Investigators scored the cases twice with a minimum of 4 weeks' interval between scoring. Scores were evaluated for intra-investigator and inter-investigator scoring reproducibility. One investigator missed (forgot to score) one case for round 1 evaluation, which left 74 staining events for reproducibility evaluations. Intra-investigator reproducibility was clinically consistent (positive versus negative) for 72 of the 74 staining interpretations - 97 %. Out of 34 intra-investigator evaluations of 2+ and 3+ positively staining cases/cell lines, there were 5 (15 %) instances of 2+/3+ intra-investigator scoring variability. Thus an investigator trained with the scoring materials provided was able to consistently interpret PATHWAY HER 2 staining of breast carcinoma cases and cell line controls, although there was still some variation in the interpretation of 2+ and 3+ staining. Inter-investigator reproducibility was evaluated for both round 1 and round 2. In round 1, 71 of 74 interpretations were clinically consistent among the five investigators - 96 %. In round 1, out of 34 inter-investigator evaluations of 2+ and 3+ positively staining cases/cell lines, there were 4 (12 %) instances of 2+/3+ inter-investigator scoring variability. In round 2, 70 of 75 interpretations were clinically consistent among the five investigators–93 %, and out of 35 inter-investigator evaluations of 2+ and 3+ positively staining cases/cell lines there was one instance (3 %), of 2+/3+ inter-investigator scoring variability. Thus investigators at different institutions trained with the scoring materials provided were able to consistently interpret PATHWAY HER 2 staining of breast carcinoma cases and cell line controls, although there was still some variation in the interpretation of 2+ and 3+ staining. This variability, however, declined with investigator experience; by round two there was only one instance of 2+/3+ inter-investigator variability.

Lot-to-lot reproducibility: To test the lot-to-lot variation of the PATHWAY HER 2, the three PATHWAY HER 2 cell line controls and two neutral

buffered formalin-fixed known positive breast carcinoma cases were run with three different GMP lots. The procedure for preparing the slides used in this study included pretreatment of the slides using antigen enhancement in an acidic buffer. Slides were then stained using a protocol consisting of 32 minute incubation of the primary antibody (PATHWAY HER 2 or negative Ig reagent), Ventana's Basic DAB detection kit, and counterstaining with hematoxylin and bluing reagent. Lot 2 exhibited a lower percentage of cells staining in the 1+ cell line control (T-47D), and a drop from 3+ to 2+ for one breast carcinoma case. All other tissues and cell lines stained uniformly with the three lots tested. No tissues or cell lines tested changed from clinically positive or clinically negative when stained with the three different lots of antibody. Thus lot-to-lot staining results are very consistent with c-erbB-2 primary antibody.

2. Immunoreactivity:

Immunoreactivity of PATHWAY HER 2 was demonstrated by a clinical study that showed appropriate staining in breast carcinoma tissue, and in-house specificity panels of normal and neoplastic tissues. The clinical study was designed to evaluate the appropriateness of PATHWAY HER 2 as an aid in the assessment of patients for whom Herceptin treatment is considered.

3. Appropriate performance for use as an aid in the assessment of Herceptin therapy.

This study examined the suitability of PATHWAY HER 2 for use as an aid in determination of treatment for Herceptin therapy. A comparative protocol was designed to examine the correlation of performance between PATHWAY HER 2 and HercepTest, a previously approved FDA diagnostic for this indication. As such, this assay was set as the gold standard. Three investigators participated in the study. Each investigator evaluated the archived neutral buffered formalin-fixed, paraffin-embedded breast carcinoma tissue blocks available at their institutions for HER-2/neu status and stratified their cases into positive and negative sub-populations. 75 cases were randomly selected from each pool for a total of 150 cases per site, 450 cases for the study.

The slides stained with HercepTest were processed and stained according to the manufacturer's instructions specified in the package insert. The procedure used for preparing the slides stained with PATHWAY HER 2 included pretreatment of the slides using antigen enhancement in an acidic buffer. Slides were then stained using a protocol consisting of 32 minute incubation of the primary antibody (PATHWAY HER 2 or negative Ig reagent), Ventana's Basic DAB detection kit, and counterstaining with hematoxylin and bluing reagent.

Results: Data were analyzed for concordance, positivity, and negativity. Concordance was evaluated in two formats: binary (positive vs. negative), and 3 X 3 (3+, 2+ > 10 %, negatives - 2+ $\leq$ 10 %, 1+, 0).

Table 5. Clinical Agreement of Ventana PATHWAY HER 2 and HercepTest

	HercepTest Negative	HercepTest Positive	Total
PATHWAY HER 2 Negative	282	17	299
PATHWAY HER 2 Positive	17	134	151
Total:	299	151	450

Concordance = 92.4 %
95% Confidence Interval = 89.6 % to 94.7 %
$p < 0.0001$

The observed agreement between the two tests was 92.4 % (416/450). The exact 95 % confidence interval was 89.6 % to 94.7 %. The null hypothesis that agreement was no greater than 75% (one-sided hypothesis) was rejected with $p < 0.0001$. The kappa statistic (chance-corrected measure of agreement) was 0.83; the null hypothesis that agreement is no better than chance was rejected with $p < 0.0001$. McNemar's Test of the hypothesis that the proportion positive by PATHWAY HER 2 was equal to the proportion positive by HercepTest could not be rejected with $p = 1.00$. Treating HercepTest as the standard, the sensitivity of PATHWAY HER 2 was 88.7 % (134/151) with 95 % confidence interval of 82.6 % to 93.3 %. The specificity was 94.3 % (282/299) with 95 % confidence interval of 91.1 % to 96.7 %.

Table 6. 3 X 3 Concordance of Ventana PATHWAY HER 2 and HercepTest

	HercepTest			
PATHWAY HER 2 ↓	**Negatives**	**2+**	**3+**	**Total:**
Negatives	282	14	3	299
2+	13	24	13	50
3+	4	14	83	101
Total:	299	52	99	450

Concordance = 86.4 %
95% Confidence Interval = 82.9 % to 89.5 %
p < 0001

The observed agreement was 86.4 % (389/450) with 95 % confidence interval of 82.9 % to 89.5 %. The null hypothesis that agreement was no greater than 75 % (one-sided hypothesis) was rejected with $p < 0.0001$.

Clinical concordance (the likelihood of a patient to be selected for treatment) between PATHWAY HER 2 and the previously approved diagnostic as an aid for determining Herceptin therapy, HercepTest is 92.4 % (95 % CI = 89.6 % - 94.7 %). The ability of each test to distinguish negatives, intermediate positives (2+) and strong positives (3+) is also in strong agreement, 86.4 % concordance (95 % CI= 82.9 % - 89.5%). The data generated in this study demonstrate that PATHWAY HER 2 is indicated as an aid in determining Herceptin therapy based upon its high concordance of staining results with HercepTest, a previously approved diagnostic test for this indication.

4. Specificity Panels

Ventana also tested normal and neoplastic tissues in which formalin-fixed, paraffin-embedded tissues were stained with PATHWAY HER 2. The procedure used for preparing the slides stained with PATHWAY HER 2 included pretreatment of the slides using antigen enhancement in an acidic buffer. Slides were then stained using a protocol consisting of 32 minute incubation of the primary antibody (PATHWAY HER 2 or negative Ig reagent), Ventana's Basic DAB detection kit, and counterstaining with hematoxylin and bluing reagent. The seventy-eight normal tissues examined included adrenal, bone marrow, breast, cerebrum, cerebellum, cervix, colon, endometrium, esophagus, heart, kidney, liver, lung, mesothelium, ovary, pancreas, peripheral nerve, prostate, salivary gland, skeletal muscle, skin, small intestine, spleen, stomach, testis, thyroid, tonsil, and thymus. Tissues not tested were parathyroid. Cytoplasmic staining was observed in the following tissues: colon (1 of 3 cases), endometrium (1 of 3 cases), kidney (1 of 2 cases), pancreas (2 of 3 cases), pituitary (1 of 2 cases), prostate (2 of 3 cases), small intestine (2 of 3 cases), stomach (2 of 3 cases), thyroid (3 of 3 cases), tonsil (3 of 3 cases), and thymus (1 of 1 case). One case of kidney and one case of tonsil showed cell membrane staining, and one case of thyroid exhibited extracellular staining. The cell membrane staining of the tonsil was limited to the squamous epithelium, a secondary component.

The nineteen neoplastic tissues examined were lung, prostate, colon, lymphoma, bladder, stomach, cervix, and ovary. Cytoplasmic staining was observed in cancer cells of the lung (3 of 3 cases), prostate (2 of 3 cases), colon (3 of 3 cases), bladder (1 of 1 case), cervix (2 of 3 cases), and ovary (2 of 2 cases) and was not considered to be a positive result.

The sensitivity of c-erbB-2 histochemistry is dependent upon the preservation of the antigen. Any improper tissue handling during fixation, sectioning, embedding, or storage which alters the antigenicity weakens the c-erbB-2 detection by Ventana's PATHWAY HER 2 and may generate false negative results.

F. Troubleshooting

1. If the c-erbB-2 positive control exhibits weaker staining than expected, check other positive controls run during the same instrument run to determine if it is due to the primary antibody or one of the common secondary reagents. Call Ventana Technical Consultation Center (800-227-2155).
2. If the c-erbB-2 positive control is negative, check to ensure that the slide has the proper bar code label. If the slide is labeled properly, check other positive controls run during the same instrument run to determine if it is due to the primary antibody or one of the common secondary reagents. Tissues may have been improperly collected,

fixed, or deparaffinized. Follow proper procedure for collection, storage, and fixation. Call Ventana Technical Consultation Center (800-227-2155).

3. If excessive background staining occurs, it may be due to residual paraffin. If this is the case, repeat deparaffinization procedure. Alternatively, high levels of endogenous biotin may be present. Pre-incubate tissue with biotin blocking reagents. (Endogenous Biotin Blocking Kit - Ventana Cat. No. 760-050).
4. If specific antibody staining is too intense, repeat run and shorten incubation time by four minute intervals or multiples thereof until desired stain intensity is achieved.
5. If tissue sections wash off slide, check to be sure slides are silanized or coated with polylysine or equivalent material. Refer to the Ventana automated slide stainer Operator's Manual for corrective action or contact Ventana Technical Consultation Center (800-227-2155).

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