PATHWAY anti-HER-2/neu (4B5) Rabbit Monoclonal Primary Antibody

REF 790-2991

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IVD Σ 50

INDICATIONS AND USE

Intended Use

This antibody is intended for in vitro diagnostic use.

Ventana Medical Systems, Inc.'s (Ventana) PATHWAY anti-HER-2/*neu* (4B5) Rabbit Monoclonal Primary Antibody (PATHWAY HER2 (4B5)) is a rabbit monoclonal antibody intended for laboratory use for the semi-quantitative detection of HER2 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 anti-HER-2/*neu* (4B5). PATHWAY anti-HER-2/*neu* (4B5) was compared to PATHWAY HER-2 (clone CB11) Primary Antibody on an independent sample set and found to provide acceptably concordant results. The actual correlation of PATHWAY anti-HER-2/*neu* (4B5) to clinical outcome has not been established.

The *VIAS* Image Analysis System is an adjunctive optional computer-assisted image analysis system functionally connected to an interactive microscope. It is intended for use as an aid to the pathologist in the detection, classification and counting of cells of interest based on marker intensity, size and shape using appropriate controls to assure the validity of the *VIAS* scores.

Summary and Explanation

PATHWAY anti-HER-2/*neu* is a rabbit monoclonal antibody (clone 4B5) directed against the internal domain of the c-erbB-2 oncoprotein (HER2). 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 HER2 may be involved in signal transduction and stimulation of mitogenic activity.[1]

Clone 4B5 has been shown to react with a 185 kD protein from SK-BR-3 cell lysates via Western blotting. SK-BR-3 is a breast carcinoma cell line, which has a 128-fold over expression of HER2 mRNA.[2] The size of the band identified correlates well with that reported by Akiyama et al for HER2 protein (185 kD).[1] Immunohistochemistry has been used to detect specific antigens in cells or tissue since 1950.[3] The use of enzymes and peroxidase as markers for immunohistochemistry was reported by Nakane and Pierce in 1967.[4] The increased sensitivity of the avidin-biotin-peroxidase detection system over the enzyme labeled antibody method was documented by Hsu et al in 1981.[5]

The HER2 protein 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 HER2.[6] Almost all cases of Paget's disease of breast[7] and up to 90 percent of cases of ductal carcinoma *in situ* of comedo type are positive.[6] The immunohistochemical detection of HER2 protein overexpression is also used as an aid in determination of patients for whom Herceptin therapy is indicated.[8]

Staining results in normal tissues, neoplastic tissues, and 322 cases of breast carcinoma with PATHWAY HER2 (4B5) were evaluated by Ventana. In the normal tissues tested, expression was consistent with the published literature in that there was no unexpected specific cytoplasmic/membrane staining, with the following exceptions: two cases of tonsil showing with epithelial cell membrane staining, one case of parathyroid, and one case of esophageal epithelium. Of the neoplastic tissues tested, cytoplasmic/membrane staining was seen in cancer cells of the breast, colon and ovary. Three hundred twenty-two (322) breast carcinomas were evaluated with VENTANA PATHWAY HER2 (4B5) in a method comparison study with PATHWAY HER-2 (CB11). There is a significant correlation of staining between these two tests. See Summary of Expected Results section for further information. Additional information on PATHWAY HER2 (4B5) can be found in the References section, 25-31.

VENTANA PATHWAY HER2 (4B5) in combination with VENTANA *i*VIEW DAB Detection Kit, utilizes biotinylated secondary antibodies to locate the bound PATHWAY HER2 (4B5) primary antibody (produced by using a synthetic peptide corresponding to a site on the internal domain of the HER2 protein). 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 HER2 (4B5) and ready-to-use *i*VIEW DAB and *ultra*View Universal DAB 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.

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.[9] Early detection and appropriate treatment therapies can significantly affect overall survival.[10] 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 their diagnosis and prognosis of disease. One important marker in breast cancer today is c-erbB-2 oncoprotein (HER2).

HER2 is an intracellular membrane protein detected in the cellular membrane.[11] It is closely related to EGFR and, like EGFR, has tyrosine kinase activity.[1] Gene amplification and the corresponding overexpression of c-erbB-2 has been found in a variety of tumors, including breast carcinomas.[11,12]

The therapeutic drug Herceptin has been shown to benefit some breast carcinoma patients by arresting, and in some cases reversing the growth of their cancer.[8] The drug is a humanized monoclonal antibody that binds to HER2 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 HER2 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 HER2 must take into consideration the fact that HER2 is expressed in both breast cancer tumors and healthy tissue, albeit at differing levels and with different patterns of expression.[13] Histological tissue preparations have the advantage of intact tissue morphology to aid in the interpretation of the HER2 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 complemented by morphological studies and proper controls and used in conjunction with other clinical and laboratory data.

Principles and Procedures

PATHWAY HER2 (4B5) is a rabbit monoclonal antibody, which binds to HER2 in paraffin-embedded tissue sections. The specific antibody can be localized by either a biotin conjugated secondary antibody formulation that recognizes rabbit immunoglobulins followed by the addition of a streptavidin-horseradish peroxidase (HRP) conjugate (*i*VIEW DAB Detection Kit) or a secondary antibody-HRP conjugate (*ultra*View Universal DAB Detection Kit). The specific antibody-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 to 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 slide.

Clinical cases should be evaluated within the context of the performance of appropriate controls. Ventana recommends the inclusion of a positive tissue control fixed and processed in the same manner as the patient specimen (for example, a weakly positive breast carcinoma). In addition to staining with PATHWAY HER2 (4B5), a second slide should be stained with CONFIRM Negative Control Rabbit Ig. For the test to be considered valid, the positive control tissue should exhibit membrane staining of the tumor cells. These components should be negative when stained with CONFIRM Negative Control Rabbit Ig. In addition, it is recommended that a negative tissue control slide (for example, a HER-2/*neu* negative breast carcinoma) be included for every batch of samples processed and run on the VENTANA automated slide stainer. This negative tissue control should be stained with PATHWAY HER2 (4B5) to ensure that the antigen enhancement and other pretreatment procedures did not create false positive staining.

The *VIAS* is an interactive histology imaging device that performs image processing using a microscope, digital color video camera, computer, and image analysis software to

acquire and analyze user-selected images on the PATHWAY HER-2/neu (4B5) stained slides.

The device is intended to aid the pathologist in their analysis by providing semi-quantitative input to supplement the pathologist's qualitative interpretation of PATHWAY HER-2/*neu* (4B5) slides. The pathologist performs the usual manual read of the HER-2/*neu* slides to assess the HER-2/*neu* expression as scored on a scale (0,1+,2+,3+) for the slide using the *VIAS* microscope. The pathologist then has the opportunity to select multiple fields of view using the *VIAS* microscope and computer for semi-quantitative analysis. The *VIAS* device processes the user-selected color images to assess the HER-2/*neu* expression using a software algorithm to aid the pathologist's qualitative read.

The pathologist makes the final call based on both the semi-quantitative and qualitative information. It is recommended that in this application of the VIAS the user follow the appropriate instructions in the VENTANA PATHWAY HER-2/*neu* (4B5) product insert and associated scoring guide.

For further information refer to the **Ventana Image Analysis System** (VIAS) Operator's Manual.

MATERIALS AND METHODS

Reagents Provided

Catalog #790-2991: PATHWAY anti-HER-2/*neu* (4B5) Primary Antibody contains sufficient reagent for 50 tests.

One 5 mL dispenser PATHWAY anti-HER-2/*neu* (4B5) Primary Antibody contains approximately 30 µg of a rabbit monoclonal antibody directed against human c-erbB-2 antigen.

The antibody is diluted in 0.05 M Tris buffered saline, 0.01 M EDTA, 0.05% Brij-35 with 0.3 % carrier protein and 0.05 % sodium azide, a preservative. There is trace fetal calf serum, approximately 0.25 %, present from the stock solution.

Total protein concentration of the reagent is approximately 16 mg/mL. Specific antibody concentration is approximately 6 µg/mL. PATHWAY anti-HER-2/*neu* (4B5) Primary Antibody is a rabbit IgG diluted from tissue culture supernatants. There is no known irrelevant antibody reactivity observed in this product.

Reconstitution, Mixing, Dilution, Titration

This antibody is optimized for use on a VENTANA automated slide stainer in combination with VENTANA *i*VIEW DAB Detection Kit and compatible with *ultra*View Universal DAB Detection Kit. No reconstitution, mixing, dilution, or titration is required.

Further dilution may result in loss of antigen staining. The user must validate any such changes. Differences in tissue processing and technical procedures in the laboratory may produce significant variability in results and require regular use of controls (see Quality Control Procedures section).

Materials and Reagents Needed But Not Provided

The following reagents and materials may be required for staining but are not provided:

1. CONFIRM Negative Control Rabbit Ig (Cat. No. 760-1029) (negative reagent control)
2. Microscope slides, Superfrost Plus [VWR Cat. No. 48311-703 or equivalent]
3. Positive and negative tissue controls (invasive breast carcinoma and normal breast tissue)
4. Bar code labels (appropriate for negative reagent control and primary antibody being tested)
5. Staining jars or baths
6. Tissue-Tek staining dishes
7. Timer (capable of 2-10 minute intervals)
8. Xylene (histological grade)
9. Ethanol or reagent alcohol (histological grade)
    - 100% solution: undiluted ethanol or reagent alcohol
    - 95% solution: mix 95 parts ethanol or reagent alcohol with 5 parts of deionized water
    - 80% solution: mix 80 parts ethanol or reagent alcohol with 20 parts deionized water
10. Deionized or distilled water
11. Biocare Medical's Decloaking Chamber (Cat. No. DC2002) (NexES IHC automated slide stainers)
12. NexES IHC, BenchMark Series automated slide stainers
13. *i*VIEW DAB (Cat. No. 760-091) or *ultra*View Universal DAB (Cat. No. 760-500) Detection Kits
14. Endogenous Biotin Blocking Kit (Cat. No. 760-050)
15. APK Wash (10X)* (Cat. No. 250-042) (NexES IHC automated slide stainers)
16. Liquid Coverslip (Low Temperature) (Cat. No. 250-009) (NexES IHC automated slide stainers)
17. EZ Prep (10X)* (Cat. No. 950-102) (BenchMark Series automated slide stainers)
18. Reaction Buffer (10X)* (Cat. No. 950-300) (BenchMark Series automated slide stainers)
19. Liquid Coverslip (High Temperature) (Cat. No. 650-010) (BenchMark Series automated slide stainers)
20. Cell Conditioning 1 (Pre-dilute) (CC1) (Cat. No. 950-124)
21. Hematoxylin II counterstain (Cat. No. 790-2208)
22. Bluing Reagent (Cat. No. 760-2037)
23. Permanent Mounting Medium (Permount, Fisher Cat. No. SP15-500 or equivalent)
24. Cover glass (sufficient to cover tissue such as VWR Cat. No. 48393-60 or equivalent)
25. Automated coverslipper (such as Tissue-Tek SCA automated coverslipper).
26. Absorbent wipes (If performing manual antigen unmasking)
27. Light microscope (20-80X) or Ventana Image Analysis System (*VIAS*)*

* As needed for specific applications.

Storage and Handling

Store at 2-8°C. Do not freeze. The user must validate any storage conditions other than those specified in the package insert.

PATHWAY HER2 (4B5) should be allowed to stand at least 30 minutes at room temperature prior to use. To ensure proper reagent delivery and stability of the antibody after every run, the cap must be replaced and the dispenser must be immediately placed in the refrigerator in an upright position.

Every antibody dispenser is expiration dated. When properly stored, the reagent is stable to the date indicated on the label. Do not use reagent beyond the expiration date for the prescribed storage method. The product has been designed to have 18 months dating after the date of manufacture.

There are no definitive signs to indicate instability of this product; therefore, positive and negative controls should be run simultaneously with unknown specimens. Your local support representative should be contacted immediately if there is an indication of reagent instability.

Specimen Collection and Preparation for Analysis

Formalin-fixed, paraffin-embedded tissues which have been antigen enhanced are suitable for use with PATHWAY HER2 (4B5) when used with VENTANA detection kits and a VENTANA automated slide stainer (see 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).[14]

Properly fixed and embedded tissues expressing the antigen will remain stable for at least 2 years if stored in a cool location (15-25°C). The Clinical Laboratory Improvement Act (CLIA) of 1988, 42CFR493.1259(b) requires 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."

Approximately 5 µm thick sections should be cut and picked up on glass slides. The slides should be Superfrost Plus or equivalent. Tissue should be air dried by placing the slides at ambient temperature overnight.[14] Studies at Ventana indicate that air dried cut tissue and cell line sections stored at 2-8°C are stable for at least 6 months. Each laboratory should validate the cut slide stability for their own procedures and environmental storage conditions.

WARNINGS AND 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 (example: xylene or formaldehyde). Do not use near open flame.
3. Do not smoke, eat or drink in areas where specimens or reagents are being handled.
4. Avoid contact of reagents with eyes and mucous membranes. If reagents come in contact with sensitive areas, wash with copious amounts of water.
5. Patient specimens and all materials contacting them should be handled as biohazardous materials and disposed of with proper precautions. Never pipette by mouth.
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. The user must validate any such change.
8. The reagents have been optimally diluted, and further dilution may result in loss of antigen staining. The user must validate any such change.
9. When used according to instructions, this product is not classified as a hazardous substance. The preservative in the reagent is sodium azide. Symptoms of overexposure to sodium azide include skin and eye irritation, and irritation of mucous membranes and upper respiratory tract. The concentration of sodium azide in this product is 0.05% and does not meet the OSHA criteria for a hazardous substance. Build up of NaN_3 may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide accumulation in plumbing.[15] Systemic allergic reactions are possible in sensitive individuals.
10. Consult local or state authorities with regard to recommended method of disposal.
11. For supplementary safety information, refer to the product Safety Data Sheet and the Symbol and Risk Phrase Guide located at www.ventana.com.

INSTRUCTIONS FOR USE

Step by Step Procedure

VENTANA primary antibodies have been developed for use on VENTANA automated slide stainers in combination with VENTANA detection kits and accessories. Recommended staining protocols for the automated slide stainers are listed below in Table 1 and Table 2. The parameters for the automated procedures can be displayed, printed and edited according to the procedure in the Operator's Manual. Other operating parameters for the automated slide stainers have been preset at the factory.

Table 1. Recommended Staining Protocols for PATHWAY anti-HER-2/neu (4B5) with *i*VIEW DAB Detection Kit.

Procedure Type	Platform or Method	
	BenchMark and BenchMark XT instrument	BenchMark ULTRA instrument
Baking	None	None
Deparaffinization	Selected	Selected
Cell Conditioning (Antigen Unmasking)	Cell Conditioning 1, Standard	ULTRA CC1, mild
Enzyme (Protease)	None required	None required
Antibody (Primary)	Approximately 32 minutes, 37°C	Approximately 24 minutes, 36°C
A/B Block (Biotin Blocking)	Required	Required
Counterstain (Hematoxylin)	Hematoxylin II, 4 minutes	Hematoxylin II, 4 minutes
Post Counterstain	Bluing, 4 minutes	Bluing, 4 minutes

Table 2. Recommended Staining Protocols for PATHWAY anti-HER-2/*neu* (4B5) with *ultra*View Universal DAB Detection Kit.

Procedure Type	Platform or Method	
	BenchMark XT instrument	BenchMark ULTRA instrument
Baking	None	None
Deparaffinization	Selected	Selected
Cell Conditioning (Antigen Unmasking)	Cell Conditioning 1, Mild	ULTRA CC1, mild
Enzyme (Protease)	None required	None required
Antibody (Primary)	Approximately 16 minutes, 37°C	Approximately 12 minutes, 36°C
Counterstain (Hematoxylin)	Hematoxylin II, 4 minutes	Hematoxylin II, 4 minutes
Post Counterstain	Bluing, 4 minutes	Bluing, 4 minutes

The procedures for staining on the VENTANA automated slide stainers are as follows. For more detailed instructions and additional protocol options, refer to your Operator's Manual.

BenchMark Series Automated Slide Stainers

1. Apply slide bar code label which corresponds to the antibody protocol to be performed.
2. Load the primary antibody, appropriate detection kit dispensers, and required accessory reagents onto the reagent tray and place them on the automated slide stainer. Check bulk fluids and waste.
3. Load the slides onto the automated slide stainer.
4. Start the staining run.
5. At the completion of the run, remove the slides from the automated slide stainer.
6. Wash in a mild dishwashing detergent to remove the coverslip solution.
7. Dehydrate, clear, and coverslip with permanent mounting media in the usual manner.
8. The stained slides should be read within two to three days of staining, and are stable for at least two years if properly stored at room temperature (15 to 25°C).

Quality Control Procedures

Cell Line System Controls

Ventana has available as a separate product four formalin-fixed cell line controls embedded in paraffin, sectioned and placed on a single charged slide (catalog # 781-2991). PATHWAY HER-2 4 in 1 Control Slides may be useful for a preliminary validation of the processing method used for staining slides with PATHWAY HER2 (4B5). These four cell line controls are characterized by *in situ* hybridization for gene copy number. When processed and stained appropriately, the cell lines should stain as described in the PATHWAY Her-2 4 in 1 Control Slide package insert. If the indicated staining is not evident in the appropriate cores, especially the 1+ and 2+ controls, the staining of the tissues should be repeated.

Table 3. Characteristics of PATHWAY HER-2 4 in 1 Control Slides.

HER2 IHC Score	Cell Line	HER2/Chr17 Ratio*
0	MDA-MB-231	1.11
1+	T47D	1.12
2+	MDA-MB-453	2.66
3+	BT-474	5.53

* HER2/Chr17 ratio is an average of three lots of PATHWAY HER-2 4 in 1 Control Slides determined using fluorescence in situ hybridization (FISH)

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 HER2

staining procedure performed. This tissue could contain both positive staining cell/tissue components and negative cell/tissue components and serve as both the positive and negative control tissue. Control tissue should be fresh autopsy/biopsy/surgical specimens prepared and fixed as soon as possible in a manner identical to test sections. Such tissue 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 preparation. 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 HER-2/*neu* is strongly positive due to the nature of the pathology (overexpression). An example of a positive control for PATHWAY HER2 (4B5) is a known weak HER-2/*neu* positive invasive breast carcinoma (for example ductal or lobular). The positive staining tissue components (cytoplasmic membrane of neoplastic cells) are used to confirm that the antibody was applied and the instrument functioned properly.

A known weak HER-2/*neu* positive invasive breast carcinoma tissue may contain both positive and negative staining cells or tissue components and may serve as both the positive and negative control tissue.

Known positive tissue controls should be utilized only for monitoring the correct performance of processed tissues and test reagents, and not as an aid in determining a specific diagnosis of patient samples.

Negative Tissue Control

The same slide used for the positive tissue control (ductal or lobular invasive breast carcinoma) 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 with the primary antibody. Alternatively, normal breast tissue is an adequate negative control tissue. Use a tissue 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 HER2 (4B5) for demonstration of HER-2/*neu*, and to provide an indication of specific background staining (false positive staining).

Negative Reagent Control

A negative reagent control must be run for every specimen to aid in the interpretation of results. A negative reagent control is used in place of the primary antibody to evaluate nonspecific staining. The slide should be stained with CONFIRM Negative Control Rabbit Ig. The incubation period for the negative reagent control should equal the primary antibody incubation period.

Unexplained Discrepancies

Unexplained discrepancies in controls should be referred to your local support representative immediately. If quality control results do not meet specifications, patient results are invalid. See the Troubleshooting section of this insert. Identify and correct the problem, then repeat the patient samples.

Assay Verification

Prior to initial use of an antibody or staining system in a diagnostic procedure, the specificity of the antibody should be verified by testing it on a series of tissues with known immunohistochemistry performance characteristics representing known positive and negative tissues (refer to the Quality Control Procedures previously outlined in this section of the product insert and to the Quality Control recommendations of the College of American Pathologists Laboratory Accreditation Program, Anatomic Pathology Checklist,[15] or the CLSI Approved Guideline[16] or both documents). These quality control procedures should be repeated for each new antibody lot, or whenever there is a change in assay parameters. Breast cancer tissues with known HER2 status are suitable for assay verification.

Interpretation of Results

The VENTANA automated immunostaining procedure causes a brown colored (DAB) reaction product to precipitate at the antigen sites localized by PATHWAY HER2 (4B5). A qualified pathologist experienced in immunohistochemical procedures must evaluate controls and qualify the stained product before interpreting results.

Positive Controls

The stained positive tissue control should be examined first to ascertain that all reagents are functioning properly. The presence of an appropriately colored reaction product within the membrane of the target cells is indicative of positive reactivity. Depending on the incubation length and potency of the hematoxylin used, counterstaining will result in a pale to dark blue coloration of cell nuclei. Excessive or incomplete counterstaining may compromise proper interpretation of results.

If the positive tissue control fails to demonstrate positive staining, any results with the test specimens should be considered invalid.

Negative Tissue Controls

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 or cellular components. The staining of normal breast is an adequate negative control tissue. Intact stromal and ductal 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.

Negative Reagent Controls

Nonspecific staining, if present, will have a diffuse appearance. Sporadic light staining of connective tissue may also be observed in tissue sections that are excessively formalin fixed. Intact cells should be used for interpretation of staining results, as necrotic or degenerated cells often stain nonspecifically.

Patient Tissue

Patient specimens should be examined last. Positive staining intensity should be assessed within the context of any background staining of the negative reagent control. 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 or tissue assayed. 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.

A qualified pathologist who is experienced in immunohistochemical procedures must evaluate positive and negative controls and qualify the stained product before interpreting results.

Scoring Conventions for the Interpretation of PATHWAY HER2 (4B5)

Breast carcinomas that are considered positive for HER-2 protein overexpression must meet 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 membrane 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 Interpretation Guide for PATHWAY HER-2/neu (4B5) for a more detailed description with photographs of staining with PATHWAY HER2 (4B5).

Table 4. Criteria for Intensity and Pattern of Cell Membrane Staining with PATHWAY HER2 (4B5).

Staining Pattern	Score (Report to Treating Physician)	HER2 Staining Assessment
No membrane staining is observed	0	Negative
Faint, partial staining of the membrane in any proportion of the cancer cells	1+	Negative
Weak complete staining of the membrane, greater than 10% of cancer cells	2+	Weakly Positive
Intense complete staining of the membrane, greater than 10% of cancer cells	3+	Positive

LIMITATIONS

General Limitations

1. Immunohistochemistry is a multiple step diagnostic process that requires specialized training in the selection of the appropriate reagents, tissue selections, fixation, processing, preparation of the immunohistochemistry 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 result from variations in fixation and embedding methods, or from inherent irregularities within the tissue.
3. Excessive or incomplete counterstaining may compromise proper interpretation of results.
4. The clinical interpretation of any positive staining, or its absence, must be evaluated within the context of clinical history, morphology and other histopathological criteria. The clinical interpretation of any staining, or its absence, must be complemented by morphological studies and proper controls as well as other diagnostic tests. It is the responsibility of a qualified pathologist to be familiar with the antibodies, reagents and methods used to interpret the stained preparation. Staining must be performed in a certified licensed laboratory under the supervision of a pathologist who is responsible for reviewing the stained slides and assuring the adequacy of positive and negative controls.
5. Ventana provides antibodies and reagents at optimal dilution for use when the provided instructions are followed. Any deviation from recommended test procedures may invalidate expected results. Appropriate controls must be employed and documented. Users who deviate from recommended test procedures must accept responsibility for interpretation of patient results.
6. This product is not intended for use in flow cytometry, performance characteristics have not been determined.
7. Reagents may demonstrate unexpected reactions in previously untested tissues. The possibility of unexpected reactions even in tested tissue groups cannot be completely eliminated because of biological variability of antigen expression in neoplasms, or other pathological tissues.[17] Contact your local support representative with documented unexpected reactions.
8. Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit nonspecific staining with horseradish peroxidase.[18]
9. False positive results may be seen because of 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 (example: liver, brain, breast, kidney) depending on the type of immunostain used.[19]
10. As with any immunohistochemistry test, a negative result means that the antigen was not detected, not that the antigen was absent in the cells or tissue assayed.

Specific Limitations

1. The antibody has been optimized as indicated in tables 1 and 2 for VENTANA platforms and detection chemistries. Because of variation in tissue fixation and processing, it may be necessary to increase or decrease the primary antibody incubation time on individual specimens. For further information on fixation variables, refer to "Immunohistochemistry Principles and Advances".[20]
2. The antibody, in combination with VENTANA detection kits and accessories, detects antigen that survives routine formalin fixation, tissue processing and sectioning. Users who deviate from recommended test procedures are responsible for interpretation and validation of patient results.
3. Bone marrow was not tested for specificity. The user should determine appropriate staining in the above tissues prior to interpretation of staining information.

SUMMARY OF EXPECTED RESULTS

The performance of the PATHWAY HER2 (4B5) Primary Antibody was evaluated through specificity, reproducibility and method comparison studies. All staining was performed using the *i*VIEW DAB Detection Kit protocol listed above on a Benchmark XT automated stainer unless otherwise specified.

1. Specificity: PATHWAY HER2 (4B5) specificity was determined by a study that showed no specific membrane staining for most normal tissues. Staining results were as follows: adrenal (0/3), breast (0/3), cerebellum (0/3), cerebrum (0/3), cervix (0/3), colon (0/3), esophagus (1/3), heart (0/2), kidney (0/3), liver (0/3), lung (0/3), mesothelial cells (0/3), ovary (0/3), pancreas (0/3), parathyroid (1/3, focal membrane staining), peripheral nerve (1/3), pituitary (0/2), prostate (1/3), salivary gland (0/3), skeletal muscle (0/3), skin (0/3), small intestine (0/3), spleen (0/3), stomach (0/3), testis (0/3), thymus (0/2), thyroid (0/3), tonsil (2/3 focal staining of surface epithelial cells), and uterus (0/3).

   PATHWAY HER2 (4B5) specificity was also determined by a study that showed no specific membrane staining in most neoplastic tissues. Staining results were as follows: breast cancer (1/4), carcinoid (0/2), colon cancer (1/3), hepatocellular cancer (0/5), leiomyoma (0/2), lung cancer (0/2), lymphoma (0/3), melanoma (0/2), ovarian cancer (1/2), pancreatic cancer (0/3), prostate cancer (0/3), renal cell cancer (0/5), sarcoma (0/2), stomach cancer (0/3), thyroid cancer (0/3), and undifferentiated cancer (0/1).

   Positive staining in tonsilar epithelieum, esophageal epithelium, prostate, peripheral nerve, parathyroid, breast cancer, colon, and ovarian cancer are consistent with published literature regarding expression of HER-2/neu.
2. Sensitivity: Sensitivity is dependent upon the preservation of the antigen. Any improper tissue handling during fixation, sectioning, embedding or storage which alters antigenicity weakens HER-2/neu protein detection by PATHWAY HER2 (4B5) and may generate false negative results.
3. Intra-run reproducibility of staining on the NexES, BenchMark, and BenchMark XT staining instrument platforms was determined by staining three slides each of five breast cancer tissues with a score of 0, 1+, 2+, and 3+ HER-2 expression. For each case, three of 3 slides stained appropriately within a run and for all instrument platforms tested. 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.
4. Inter-run and inter-platform reproducibility of staining was determined by staining three slides each of five breast cancer tissues with scores of 0, 1+, 2+, and 3+ HER-2 expression on three different instrument runs across the NexES, BenchMark, and BenchMark XT instrument platforms. For each case, nine of 9 slides stained appropriately over three instrument runs and across all instrument platforms tested. Users should verify between run reproducibility results by staining several sets of serial sections with low, medium and high antigen density on different days.
5. BenchMark XT Inter-laboratory staining and Inter-reader scoring reproducibility: Three laboratories, from separate institutions in the United States, participated in the inter-laboratory reproducibility study. Cut slides of 40 neutral buffered formalin-fixed invasive breast carcinoma cases [10 each from each HER-2 binning category (0-1+, 2+, 3+)] and six (6) PATHWAY HER-2 4 in 1 Control Slides were shipped to each of the sites for staining on a VENTANA BenchMark XT automated slide staining device using the recommended staining protocol. Controls included the PATHWAY HER-2 4 in 1 Control Slides and a second slide of each case stained with negative Ig reagent. No sites experienced invalid runs, based upon the performance of the controls. The results were analyzed by Ventana. Thirty-four of forty (34/40) slides exhibited similar staining intensity across staining sites. Six samples (6/40 or 15%) varied by no more than 1 intensity level. Three (3/6) samples varied between 0 and 1+, which are both considered to be negative. Two samples (2/40 or 5%) varied between 2+ and 3+, and one sample (1/40) varied between 1+ and 2+.
6. BenchMark XT Inter-reader scoring reproducibility: In all of the 40 cases (100%), a minimum of 2 of 3 pathologists agreed.
7. Lot-to-Lot reproducibility was determined by automated staining of 5 breast cancer tissues with scores of 0, 1+, 2+, and 3+ HER2 expression with 3 lots of PATHWAY HER2 (4B5). Stained tissues were scored on a 0 to 3+ scale by three qualified readers. There was 100% agreement between lots and readers for the 3 slides and 5 tissues stained.
8. Comparison Studies of PATHWAY HER2 (4B5) rabbit monoclonal antibody to PATHWAY HER-2 (CB11) mouse monoclonal antibody: Summary of Studies Performed. A method comparison study was conducted to examine the correlation of PATHWAY HER2 (4B5) to PATHWAY HER-2 (CB11) and PathVysion Her-2 FISH, both previously approved FDA diagnostic tests. Six investigators participated in the study. Two sets of three different investigators evaluated two independent cohorts (Cohort 1: n=144, Cohort 2: n=178) using known breast cancer cases stained with HER-2 CB11 and HER2 4B5. FISH data was obtained from patient history. A consensus score from the three readers for each antibody was created for each case to reduce intra-reader variability known to exist with HER-2 scoring.[22,23,24] A total of 322 cases were evaluated. The slides stained with PATHWAY HER-2 (CB11) were processed and stained according to the manufacturer's instructions specified in the VENTANA CB11 package insert. There was an average of approximately one year between staining and reading of the

CB11 stained slides. Since scores from one of the six readers was outside of the confidence interval, data from the two cohorts are presented as follows.

Inter-pathologist Reproducibility of Comparison Studies Specimens

Table 5. Cohort 1-Consensus IHC Scores of Three Pathologists.

	CB11 Score			
4B5 Score	3+	2+	0, 1+	Total
3+	29	24	5	58
2+	2	13	17	32
0, 1+	0	0	53	53
Total	31	37	75	143

Cohort 1: Performance characteristics for 3 x 3 Presentation.

Overall agreement is 29+13+53/143=66.4% (95% C.I. = 38.6%, 59.7%).

Cohort 1: Performance characteristics for 2 x 2 Presentation (HER-2 antibody positive (2+ and 3+) and negative (0+ and 1+) scores are combined).

- Positive percent agreement is 29+2+24+13/31+37 =100% (95% C.I. %= 97.5% - 100%).
- Negative percent agreement is 53/75 = 70.7% (95% C.I. = 58.5% - 80.1%).
- Overall agreement is 29+24+2+13+53/143=84.7% (95% C.I. = 78.2% - 90.0).

Table 6. Cohort 2- Consensus IHC Scores of Three Pathologists.

	CB11 Score			
4B5 Score	3+	2+	0, 1+	Total
3+	72	1	0	73
2+	1	12	5	18
0, 1+	0	7	80	87
Total	73	20	85	178

Cohort 2: Performance characteristics for 3 x 3 Presentation.

Overall agreement is 72+12+80/178=92.1% (95% C.I. = 80.1%, 93.1%).

Cohort 2: Performance characteristics for 2 x 2 Presentation (HER-2 antibody positive (2+ and 3+) and negative (0+ and 1+) scores are combined).

- Positive percent agreement is 72+12+1+1/73+20 = 92.5% (95% C.I. = 85.2% - 96.9%).
- Negative percent agreement is 80/85 = 94.1% (95% C.I. = 86.8% - 98.1%).
- Overall agreement is 72+12+1+1+80/178=93.3% (95% C.I. = 88.5% - 96.4%).

Table 7. Cohort 1- Consensus CB11 IHC Scores of Three Pathologists Compared to FISH.

	FISH Result		
CB11 Score	Positive	Negative	Total
3+	32	0	32
2+	32	5	37
0, 1+	22	53	75
Total	86	58	144

Cohort 1: Performance characteristics for CB11 and FISH, 2 x 2 Presentation (where scores of 2 and 3 are considered positive).

- Positive percent agreement is 32+32/86= 74.4% (95% C.I. = 63.8% - 83.2%).
- Negative percent agreement is 53/58 = 91.4% (95% C.I. = 80.9% - 97.1%).
- Overall agreement is 32+32+53/144=81.2% (95% C.I. = 73.9% - 87.2%).

Table 8. Cohort 1- Consensus 4B5 IHC Scores of Three Pathologists Compared to FISH.

	FISH Result		
4B5 Score	Positive	Negative	Total
3+	55	3	58
2+	25	8	33
0, 1+	6	47	53
Total	86	58	144

Cohort 1: Performance characteristics for 4B5 and FISH, 2 x 2 Presentation (where scores of 2 and 3 are considered positive).

- Positive percent agreement is 55+25/ 86= 93.0% (95% C.I. = 87.9% - 96.3%).
- Negative percent agreement is 47/58 = 81.0% (95% C.I. = 73.4% - 86.0%).
- Overall agreement is 55+25+47/144=88.2% (95% C.I. = 82.1% - 92.2%).

Table 9. Cohort 2- Consensus CB11 IHC Scores of Three Pathologists Compared to FISH.

	FISH Result		
CB11 Score	Positive	Negative	Total
3+	72	1	73
2+	13	7	20
0, 1+	8	77	85
Total	93	85	178

Cohort 2: Performance characteristics for CB11 and FISH, 2 x 2 Presentation (where scores of 2 and 3 are considered positive).

- Positive percent agreement is 72+13/ 93= 91.3% (95% C.I. = 85.0% - 96.7%).
- Negative percent agreement is 77/85 = 90.6% (95% C.I. = 83.9% - 96.3%).
- Overall agreement is 72+13+77/178=91.0% (95% C.I. = 86.5% - 94.9%).

Table 10. Cohort 2- Consensus 4B5 IHC Scores of Three Pathologists: Compared to FISH.

	FISH Result		
4B5 Score	Positive	Negative	Total
3+	72	1	73
2+	11	7	18
0, 1+	10	77	87
Total	93	85	178

Cohort 2: Performance characteristics for 4B5 and FISH, 2 x 2 Presentation (where scores of 2 and 3 are considered positive).

- Positive percent agreement is 72+11/ 93= 89.2% (95% C.I. = 82.5% - 95.1%)
- Negative percent agreement is 77/85 = 90.6% (95% C.I. = 84.0% - 96.4%)
- Overall agreement is 72+11+77/178=90.0% (95% C.I. = 85.4% - 93.6%)

Inter-pathologist Reproducibility of Comparison Studies Specimens

Since it is well known that different pathologists may have different interpretations of immunohistochemistry slides, three pathologists were employed for each of the two cohorts (for a total of 6 pathologists) to read all samples. A two-out-of-three rule was used to adjudicate the final results. Below is a summary of the variable results obtained by the three pathologists of the comparison study samples for each cohort (Cohort 1: n=178, Cohort 2: n=144).

Table 11. Cohort 1: 4B5 Scoring for the Three Pathologists.

	4B5 Score		
HER2 Score	**Investigator 1**	**Investigator 2**	**Investigator 3**
3+	72	70	73
2+	22	19	18
0,1+	80	89	87
Total	174	178	178

Note: A total of 3 samples varied by more than one grade level (i.e. 0, 2+) when evaluated by the three pathologists.

Sample 1: One pathologist scored 2+, two pathologists scored 0+.

Sample 2: One pathologist scored 0+ two pathologists scored 2+.

Sample 3: One pathologist scored 0+, the second scored 1+, and the third scored 2+.

Table 12. Cohort 1: CB11 Scoring for the Three Pathologists.

	CB11 Score		
HER2 Score	**Investigator 1**	**Investigator 2**	**Investigator 3**
3+	72	75	73
2+	22	22	18
0,1+	80	81	87
Total	174	178	178

Note: A total of 1 sample varied by more than one grade level (i.e.1 - 3+) when evaluated by the three pathologists.

Sample 1: One pathologist scored 1+, the second scored 2+, and the third scored 3+.

Table 13. Cohort 2: 4B5 Scoring for the Three Pathologists.

	4B5 Score		
HER2 Score	**Investigator 4**	**Investigator 5**	**Investigator 6**
3	59	65	50
2	30	28	39
0,1	52	51	55
Total	141	144	144

Note: A total of 6 samples varied by more than one grade level (e.g. 0, 3+) when evaluated by the three pathologists.

Sample 1: One pathologist scored 0+, the second scored 0+, and the third scored 2+.

Sample 2: One pathologist scored 1+, the second scored 1+, and the third scored 3+.

Sample 3: One pathologist scored 0+, the second scored 2+, and the third pathologist scored 2+.

Sample 4 and 5: One pathologist scored 0+, the second scored 2+, and the third scored 2+.

Sample 6: One pathologist scored 0+, the second scored 3+, and the third scored 3+.

Table 14. Cohort 2: CB11 Scoring for the Three Pathologists.

	CB11 Score		
HER2 Score	**Investigator 4**	**Investigator 5**	**Investigator 6**
3+	31	37	28
2+	38	32	47
0,1+	75	75	69
Total	144	144	144

Note: A total of 8 samples varied by more than one grade level (i.e.. 0 - 2+) when evaluated by the three Pathologists.

Samples 1-6: one pathologist scored 0+, the second scored 1+, and the third scored 2+.

Samples 7 and 8: one pathologist scored 0+, the second scored 2+, and the third scored 2+.

Following is a tabulation of the ranges of percent agreements across pairs of pathologists (three pairs for each cohort).

Table 15. Ranges of 2X2* Agreements for the Three Pathologists.

	Overall Percent Agreement	Positive Percent Agreement	Negative Percent Agreement
4B5 vs. CB11			
Cohort 1	82.6 – 86.9%	97.3 – 100.0%	68.0% - 75.4%
Cohort 2	88.2 – 95.5%	87.6 – 95.6%	86.1 – 95.4%
4B5 vs. FISH			
Cohort 1	86.8 – 88.2%	90.7 – 94.2%	79.3 – 81.0%
Cohort 2	87.4 – 89.9%	88.2 – 90.0%	84.5 – 91.8%
CB11 vs. FISH			
Cohort 1	79.9 – 84.0%	73.3 – 80.2%	89.7 – 89.7%
Cohort 2	84.8% - 93.3%	86.7 – 92.5%	82.7 – 94.1%

* 0, 1+ = Negative. 2+ and 3+ = Positive

Conclusion: Data from these studies indicated that the PATHWAY HER2 (4B5) primary antibody was specific and reproducible in its ability to locate appropriate membrane staining for normal and neoplastic tissues. The method comparison data demonstrated that PATHWAY HER2 (4B5) primary antibody is indicated as an aid in the assessment of breast cancer patients for whom Herceptin treatment is considered.

Performance characteristics on BenchMark ULTRA instrument using *i*VIEW DAB Detection Kit or *ultra*View Universal DAB Detection Kit.

9. BenchMark ULTRA Inter-laboratory staining and inter-day reproducibility: Three laboratories, from separate institutions in the United States, participated in the inter-laboratory reproducibility study. Cut slides of 48 FFPE invasive breast carcinoma cases [12 each from each HER-2 binning category (0, 1+, 2+, 3+)] and 1 pair of PATHWAY HER-2 4 in 1 Control Slides per each of 12 staining runs were distributed to study sites for staining on a VENTANA BenchMark ULTRA automated slide staining device using the recommended staining protocol and *ultra*View Universal DAB Detection Kit. Controls included the PATHWAY HER-2 4 in 1 Controls Slides and a second slide of each case stained with negative Ig reagent. Pathologists, blinded to case status, evaluated the slides and provided a clinical score (i.e., 0, 1+, 2+, 3+). The results were analyzed by Ventana. Using standard nomenclature for 2x2 tables, average positive agreement (APA) across sites was calculated as [2a/(2a+b+c)] and average negative agreement (ANA) was calculated as [2d/(2d+b+c)]. Across all sites, the inter-site APA based on clinical assessment (positive, negative) was 90.0% (108/120) and the ANA was 92.9% (156/168). For pair-wise comparisons of sites, APA was calculated as a/(a+c) and ANA was calculated as d/(b+d). The inter-site APA rates were 93.0% (40/43), 87.2% (34/39), and 89.5% (34/38) for Site A vs. Site B, Site A vs. Site C, and Site B vs. Site C, respectively. The inter-site ANA rates were 94.3% (50/53), 91.2% (52/57), and 93.1% (54/58) for Site A vs. Site B, Site A vs. Site C, and Site B vs. Site C, respectively.
10. The following Table 16, Table 17 and Table 18 are 3x3 presentations of results for each reader based on clinical score where 2+ and 3+ were separated:

Table 16. Site A vs. Site B Inter-laboratory Agreement Rates 3x3 Analysis – PATHWAY HER2 (4B5) BenchMark ULTRA instrument with *ultra*View Universal DAB Detection Kit.

	Site B			
Site A	**3+**	**2+**	**0, 1+**	**Total**
3+	12	2	0	14
2+	0	6	2	8
0, 1+	0	1	25	26
Total	12	9	27	48
Overall percent agreement (OPA): n/N (%)			43/48 (**89.6**)	

Table 17. Site A vs. Site C Inter laboratory Agreement Rates 3x3 Analysis – PATHWAY HER2 (4B5) BenchMark ULTRA instrument with *ultra*View Universal DAB Detection Kit.

	Site C			
Site A	**3+**	**2+**	**0, 1+**	**Total**
3+	12	1	1	14
2+	0	4	4	8
0, 1+	0	0	26	26
Total	12	5	31	48
Overall percent agreement (OPA): n/N (%)			42/48 (**87.5**)	

Table 18. Site B vs. Site C Inter laboratory Agreement Rates 3x3 Analysis – PATHWAY HER2 (4B5) BenchMark ULTRA instrument with *ultra*View Universal DAB Detection Kit.

	Site C			
Site B	**3+**	**2+**	**0, 1+**	**Total**
3+	12	0	0	12
2+	0	5	4	9
0, 1+	0	0	27	27
Total	12	5	31	48
Overall percent agreement (OPA): n/N (%)			44/48 (**91.7**)	

11. BenchMark ULTRA Inter day staining reproducibility: The inter day reproducibility (IDR) portion of the study included 12 cases with an intended distribution of approximately three (3) cases at each clinical score (0, 1+, 2+, 3+). In total, the five runs on the BenchMark ULTRA automated slide staining device at the single institution (Site C) conducting the IDR portion of the study took place over a minimum of 20 days, such that no two staining days were consecutive. The IDR APA and ANA rates based on clinical assessment of PATHWAY HER2 (4B5) staining at Site C across all days were both 100%. The overall percent agreement rates (OPA) rates for inter-day comparisons based on clinical scores were 100% for each of the day-to-day comparisons and for all days combined.
12. Comparison study of BenchMark ULTRA to BenchMark XT staining platforms:

    Two staining laboratories and three reading sites in the United States participated in the platform comparison study. Cut slides of 280 FFPE invasive breast carcinoma cases [approximately 70 cases from each HER-2 binning category (0, 1+, 2+, 3+)] were randomly distributed to two staining sites (140 cases to each site) for staining on a VENTANA BenchMark XT and a VENTANA BenchMark ULTRA automated slide staining device using the respective recommended staining protocols and *ultra*View Universal DAB Detection Kit. Controls included the PATHWAY HER-2 4 in 1 Controls Slides and a second slide of each case stained with negative Ig reagent. Stained cases from Site 1 and Site 2 were divided into four slide sets and provided, one set at a time, to three different qualified readers (pathologists), one reader at Site 1, one at Site 2, and one at Site 3. The pathologists, blinded to case status and staining platform, evaluated all four sets of slides and provided a clinical score (i.e. 0, 1+, 2+, 3+) for each case. The results were analyzed by Ventana. The PPA rates (and lower bound of the two-sided 95% confidence intervals) for PATHWAY HER2 (4B5) antibody staining on the BenchMark ULTRA instrument versus the BenchMark XT instrument based on positive versus negative clinical assessment were 91.6% (85.9), 91.2% (85.3), and 94.9% (89.3) for Reader A, B, and C, respectively. The NPA rates (and lower bound of the two-sided 95% confidence intervals) for PATHWAY HER2 (4B5) antibody staining on the Benchmark ULTRA instrument versus the BenchMark XT instrument based on positive versus negative clinical assessment were 91.9% (85.8), 93.8% (88.3), and 99.3% (96.3) for Reader A, B, and C, respectively. The OPA between the PATHWAY HER2 (4B5) assay using BenchMark ULTRA instrument versus BenchMark XT instrument based on 2x2 analysis of positive versus negative clinical assessment was 91.8%, 92.5%, and 97.4% per Reader A, B, and C, respectively. The 3x3 presentation of inter-platform agreement rates for each reader based on clinical score (0/1+, 2+, 3+) are shown in Table 19, Table 20, and Table 21 below:

Table 19. BenchMark ULTRA vs. BenchMark XT Inter-Platform Agreement Rates 3x3 Analysis – Reader A.

BenchMark ULTRA instrument	BenchMark XT instrument			
Reader A	**3+**	**2+**	**0, 1+**	**Total**
3+	84	11	1	96
2+	8	28	9	45
0, 1+	4	8	114	126
Total	96	47	124	267
Overall percent agreement: n/N (%) (95% CI)			226/267 (**84.6**) (79.8-88.5)	

Table 20. BenchMark ULTRA vs. BenchMark XT Inter-Platform Agreement Rates 3x3 Analysis – Reader B.

BenchMark ULTRA instrument	BenchMark XT instrument			
Reader B	**3+**	**2+**	**0, 1+**	**Total**
3+	64	2	1	67
2+	3	56	7	66
0, 1+	2	10	122	134
Total	69	68	130	267
Overall percent agreement: n/N (%) (95% CI)			242/267 (**90.6**) (86.5-93.6)	

Table 21. BenchMark ULTRA vs. BenchMark XT Inter-Platform Agreement Rates 3x3 Analysis – Reader C.

BenchMark ULTRA instrument	BenchMark XT instrument			
Reader C	**3+**	**2+**	**0, 1+**	**Total**
3+	64	1	0	65
2+	2	45	1	48
0, 1+	0	6	148	154
Total	66	52	149	267
Overall percent agreement: n/N (%) (95% CI)			257/267 (**96.3**) (93.2-98.0)	

13. Inter-pathologist Reproducibility of Platform Comparison Study Specimens: Positive and negative agreement rates with two-sided score 95% confidence intervals were calculated for the six possible pairwise comparisons between readers for each platform.

For BenchMark ULTRA instrument, PPA rates for Reader A vs. B, A vs. C, B vs. C, B vs. A, C vs. A, and C vs. B were 94.7% (126/133), 98.2% (111/113), 98.2% (111/113), 89.4% (126/141), 78.7% (111/141), and 83.5% (111/133), respectively. NPA rates for Reader A vs. B, A vs. C, B vs. C, B vs. A, C vs, A, and C vs. B were 88.8% (119/134), 80.5% (124/154), 85.7% (132/154), 94.4% (119/126), 98.4% (124/126), and 98.5% (132/134), respectively. The OPA rate was highest between Reader A and Reader B (91.8%) and lower between Reader B and Reader C (91.0%) and Reader A and Reader C (88.8%).

For BenchMark XT instrument, PPA rates for Reader A vs. B, A vs. C, B vs. C, B vs. A, C vs. A, and C vs. B were 94.9% (130/137), 98.3% (116/118), 98.3% (116/118), 90.9% (130/143), 81.1% (116/143), and 84.7% (116/137), respectively. NPA rates for Reader A vs. B, A vs. C, B vs. C, B vs. A, C vs. A, and C vs. B were 90.0% (117/130), 81.9% (122/149), 85.9% (128/149), 94.4% (117/124), 98.4% (122/124), and 98.5% (128/130), respectively. The OPA rate was highest between Reader A and Reader B (92.5%) and lower between Reader B and Reader C (91.4 %) and Reader A and Reader C (89.1%).

14. Comparison study of *i*VIEW DAB Detection Kit to *ultra*View Universal DAB Detection Kit:

The Site 1 cohort of 140 FFPE invasive breast carcinoma cases [approximately 35 cases from each HER-2 binning category (0, 1+, 2+, 3+)] was used in a comparison study of *i*VIEW DAB Detection Kit to *ultra*View Universal DAB Detection Kit when staining with PATHWAY HER2 (4B5) on BenchMark ULTRA automated slide staining device. A single staining laboratory and three reading sites in the United States participated in the detection comparison study. For PATHWAY HER2 (4B5) antibody staining on the BenchMark ULTRA instrument the PPA rates between results obtained using *i*VIEW DAB Detection Kit and *ultra*View Universal DAB Detection Kit methods based on clinical assessment (positive, negative) were 95.8% (68/71), 96.9% (63/65), and 96.5% (55/57) for Readers A, B, and C, respectively and the NPA rates between detection methods were 90.8% (59/65), 91.5% (65/71), and 97.5% (77/79) for Readers A, B, and C, respectively. The OPA rates between detection kits were 93.4% (127/136), 94.1% (128/136), and 97.1% (132/136) for Readers A, B, and C, respectively. The 3x3 presentation of detection comparison agreement rates for each reader based on clinical score (0/1+, 2+, 3+) are shown in Table 22, Table 23, and Table 24 below:

Table 22. Reader A, *i*VIEW DAB Detection Kit vs. *ultra*View Universal DAB Detection Kit Agreement Rates 3x3 Analysis – PATHWAY HER2 (4B5) Staining on BenchMark ULTRA instrument.

*i*VIEW DAB Detection Kit	*ultra*View Universal DAB Detection Kit			
Reader A	**3+**	**2+**	**0, 1+**	**Total**
3+	43	5	0	48
2+	3	17	6	26
0, 1+	0	3	59	62
Total	46	25	65	136
Overall percent agreement: n/N (%) (95% CI)			119/136 (**87.5**) (80.9-92.0)	

Table 23. Reader B, *i*VIEW DAB Detection Kit vs. *ultra*View Universal DAB Detection Kit Agreement Rates 3x3 Analysis – PATHWAY HER2 (4B5) Staining on BenchMark ULTRA instrument.

*i*VIEW DAB Detection Kit	*ultra*View Universal DAB Detection Kit			
Reader B	**3+**	**2+**	**0, 1+**	**Total**
3+	32	0	0	32
2+	0	31	6	37
0, 1+	1	1	65	67
Total	33	32	71	136
Overall percent agreement: n/N (%) (95% CI)			128/136 (**94.1**) (88.8-97.0)	

Table 24. Reader C, *i*VIEW DAB Detection Kit vs. *ultra*View Universal DAB Detection Kit Agreement Rates 3x3 Analysis – PATHWAY HER2 (4B5) Staining on BenchMark ULTRA instrument.

*i*VIEW DAB Detection Kit	*ultra*View Universal DAB Detection Kit			
Reader C	**3+**	**2+**	**0, 1+**	**Total**
3+	32	0	0	32
2+	0	23	2	25
0, 1+	0	2	77	79
Total	32	25	79	136
Overall percent agreement: n/N (%) (95% CI)			132/136 (**97.1**) (92.7-98.9)	

15. Inter-pathologist Reproducibility of Detection Comparison Study Specimens:
Positive and negative agreement rates with two-sided score 95% confidence intervals were calculated for the six possible pairwise comparisons between readers for each method.
For *i*VIEW DAB Detection Kit, PPA rates for Reader A vs. B, A vs. C, B vs. C, B vs. A, C vs. A, and C vs. B were 100.0% (69/69), 98.2% (56/57), 96.5% (55/57), 93.2% (69/74), 75.7% (56/74), and 79.7% (55/69) respectively. NPA rates for Reader A vs. B, A vs. C, B vs. C, B vs. A, C vs, A, and C vs. B were 92.5% (62/67), 77.2% (61/79), 82.3% (65/79), 100.0% (62/62), 98.4% (61/62), and 97.0% (65/67) respectively. The overall agreement rate was highest between Reader A and Reader B (96.3%) and lower between Reader A and Reader C (86.0%) and Reader B and Reader C (88.2%).
For *ultra*View Universal DAB Detection Kit, PPA rates for Reader A vs. B, A vs. C, B vs. C, B vs. A, C vs. A, and C vs. B were 96.9% (63/65), 98.2% (56/57), 98.2% (56/57), 88.7% (63/71), 78.9% (56/71), and 86.2% (56/65), respectively. NPA rates for Reader A vs. B, A vs. C, B vs. C, B vs. A, C vs. A, and C vs. B were 88.7% (63/71), 81.0% (64/79), 88.6% (70/79), 96.9% (63/65), 98.5% (64/65), and 98.6% (70/71), respectively. The overall agreement rates were similar for each pair of readers, 92.6% (126/136), 88.2% (120/136), and 92.6% (126/136) for Reader A vs. B, Reader A vs. C, and Reader B vs. C, respectively.

Conclusion: Data from these studies indicated that the PATHWAY HER2 (4B5) primary antibody was specific and reproducible in its ability to locate appropriate membrane staining for normal and neoplastic tissues. The method comparison data demonstrated that PATHWAY HER2 (4B5) primary antibody is indicated as an aid in the assessment of breast cancer patients for whom Herceptin treatment is considered.

TROUBLESHOOTING

1. If the positive control exhibits weaker staining than expected, other positive controls run during the same instrument run should be checked to determine if it is because of the primary antibody or one of the common secondary reagents.
2. If the positive control is negative, it should be checked to ensure that the slide has the proper bar code label. If the slide is labeled properly, other positive controls run on the same instrument run should be checked to determine if it is because of the primary antibody or one of the common secondary reagents. Tissues may have been improperly collected, fixed or deparaffinized. The proper procedure should be followed for collection, storage and fixation.
3. If all of the paraffin has not been removed, there may be no staining. The deparaffinization procedure should be repeated.
4. If specific antibody staining is too intense, the run should be repeated with incubation time shortened by 4 minute intervals until the desired stain intensity is achieved.
5. If tissue sections wash off the slide, slides should be checked to ensure that they are positively charged.
6. For corrective action, refer to the Step By Step Procedure section, the automated slide stainer Operator's Manual or contact your local support representative.

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