VENTANA anti-HER2/neu (4B5) Rabbit Monoclonal Primary Antibody

REF 790-4493

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IVD Σ 50

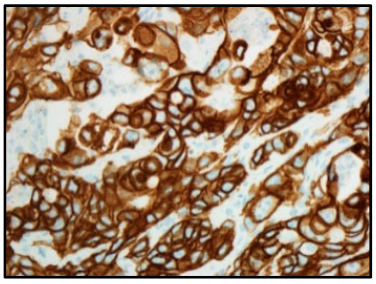

Figure 1. VENTANA anti-HER2 (4B5) antibody 3+ staining in gastric carcinoma.

INTENDED USE

VENTANA anti-HER2/neu (4B5) Rabbit Monoclonal Primary Antibody is intended for the semi-quantitative detection of HER2 antigen in sections of formalin-fixed, paraffin-embedded normal and neoplastic breast and gastric tissue stained on a BenchMark IHC/ISH instrument.

This product is indicated as an aid in the assessment of breast and gastric cancer patients for whom Herceptin® (trastuzumab) treatment is considered and for breast cancer patients for whom KADCYLA® (ado-trastuzumab emtansine) or PERJETA® (pertuzumab) treatments are being considered.

This product should be interpreted by a qualified pathologist in conjunction with histological examination, relevant clinical information, and proper controls.

This antibody is intended for in vitro diagnostic (IVD) use.

SUMMARY AND EXPLANATION

VENTANA anti-HER2/neu (4B5) Rabbit Monoclonal Primary Antibody (VENTANA anti-HER2 (4B5) antibody) is a rabbit monoclonal antibody (clone 4B5) directed against the internal domain of human epidermal growth factor receptor 2 (HER2). HER2 was cloned and characterized by Akiyama et al in 1986.[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 overexpression of HER2 mRNA.[2] The size of the band identified correlates well with that reported for HER2 protein (185 kD).[1] Immunohistochemistry (IHC) experiments with transfected cell lines (HEK293) have shown that clone 4B5 stains cells transfected with HER2 and cells transfected with HER4. No staining of cells transfected with HER1 or HER3 was observed. Western blot data with recombinant HER4 protein also indicated that clone 4B5 recognizes a HER4 epitope.

HER2 is an approximately 185 kD transmembrane receptor tyrosine kinase, which is structurally similar to epidermal growth factor receptor.[3,4] Gene amplification and the corresponding overexpression of HER2 has been found in a variety of tumors, including breast and gastric carcinomas.[3,4,5] Protein overexpression, due to amplification of the *HER2* gene, is the primary driver of HER2 mediated tumorigenesis.[3] Excess HER2 protein expression at the cell membrane enhances signal transduction, which upregulates proliferation and differentiation and ultimately causes tumor formation.[3,4,5]

Between 15-30% of invasive ductal cancers are positive for HER2 protein overexpression and/or gene amplification.[6,7] Almost all cases of Paget's disease of breast and up to 90% of cases of ductal carcinoma in situ of comedo type are positive.[6,8] HER2 protein overexpression and/or gene amplification occurs in gastric and gastroesophageal junction adenocarcinoma.[9,10,11] A wide range of HER2 overexpression frequency has been reported across published studies. However, one of the largest screening datasets which included 3,803 patients with gastric and gastroesophageal junction adenocarcinoma reported that 22 % of patients tested positive for HER2 protein overexpression or gene amplification.[12]

CLINICAL SIGNIFICANCE

Breast cancer is the most common carcinoma occurring in women, and the second leading cause of cancer related death.[4,6] In North America, a woman's chance of contracting breast cancer is one in eight.[4] Early detection and appropriate treatment therapies can significantly affect overall survival.[13,14] Small tissue samples may be easily used in routine 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 the oncoprotein HER2.[15-18] The therapeutic drugs Herceptin (trastuzumab), PERJETA (pertuzumab) and KADCYLA (ado-trastuzumab emtansine/ trastuzumab emtansine) have been shown to benefit some breast carcinoma patients by arresting, and in some cases reversing the growth of their cancer.[15-19] The drugs are humanized monoclonal antibodies that bind to HER2 protein on cancer cells.[7,15,18,19] In vitro diagnostics for the evaluation of HER2 status in breast cancer patients are important to aid the clinician in determination of HER2-targeted therapy.[15-18] The IHC-based detection of HER2 protein expression is used as an aid in the assessment of breast cancer patients for whom the HER2 targeted treatments Herceptin (trastuzumab), PERJETA (pertuzumab) or KADCYLA (trastuzumab emtansine) are being considered.

Gastric cancer is the fifth most common cancer and a leading cause of cancer-related death globally.[10] Surgery is the most common treatment for gastric cancer.[11,20] However, most gastric cancer cases are detected at an advanced stage and the surgery is often difficult to perform.[11,20] Chemotherapy is used for treating advanced gastric cancer even though the survival of cancer patients is very low. [11,20] The HER2 targeted therapy trastuzumab is a mainstay in the management of invasive breast carcinoma and has therapeutic value in the management of gastric cancer patients overexpressing the receptor.[9,11] Demonstration of *HER2* gene amplification and/or protein overexpression is essential for selecting patients for trastuzumab therapy.[9,11] Clinical studies have shown that breast or gastric cancer patients with high HER2 protein overexpression and/or gene amplification benefit most from trastuzumab.[9] The IHC-based detection of HER2 protein expression is used as an aid in the assessment of gastric cancer patients for whom Herceptin (trastuzumab) treatment is being considered.

Interpretation of the results of any detection system for HER2 must take into consideration the fact that HER2 is expressed in both breast and gastric cancer tumors and healthy tissue, albeit at differing levels and with different patterns of expression.[10,21] Histological tissue preparations have the advantage of intact tissue morphology to aid in the interpretation of the HER2 positivity of the sample.

PRINCIPLE OF THE PROCEDURE

VENTANA anti-HER2 (4B5) antibody is a rabbit monoclonal antibody, which binds to HER2 in formalin-fixed, paraffin-embedded (FFPE) 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 instrument washes the sections to stop the reaction and to remove unbound material that would hinder the desired reaction in subsequent steps. The instrument 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. The inclusion of a positive tissue control fixed and processed in the same manner as the patient specimen is recommended (for example, a weakly positive breast or gastric carcinoma). In addition to staining with VENTANA anti-HER2 (4B5) antibody, 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 (for example, a HER2 negative breast or gastric carcinoma) be included for every batch of samples processed and run on the BenchMark IHC/ISH instrument. This negative tissue control should be stained with VENTANA anti-HER2 (4B5) antibody to ensure that the antigen enhancement and other pretreatment procedures did not create false positive staining.

MATERIAL PROVIDED

VENTANA anti-HER2 (4B5) antibody contains sufficient reagent for 50 tests.

One 5 mL dispenser of VENTANA anti-HER2 (4B5) antibody contains approximately 30 µg of a rabbit monoclonal antibody directed against human HER2 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.

Specific antibody concentration is approximately 6 µg/mL. VENTANA anti-HER2 (4B5) antibody is a rabbit IgG diluted from tissue culture supernatants.

Refer to the appropriate VENTANA detection kit method sheet for detailed descriptions of: Principle of the Procedure, Material and Methods, Specimen Collection and Preparation for Analysis, Quality Control Procedures, Troubleshooting, Interpretation of Results, and Limitations.

MATERIALS REQUIRED BUT NOT PROVIDED

Staining reagents, such as VENTANA detection kits and ancillary components, including negative and positive tissue control slides, are not provided.

Not all products listed in the method sheet may be available in all geographies. Consult your local support representative.

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

1. Recommended control tissue
2. Microscope slides, positively charged
3. Rabbit Monoclonal Negative Control Ig (Cat. No. 790-4795 / 06683380001)
4. *ultra*View DAB Detection Kit (Cat. No. 760-500 / 05269806001)
5. *i*VIEW DAB Detection Kit (Cat. No. 760-091 / 05266157001 (50 test))
6. EZ Prep Concentrate (10X) (Cat. No. 950-102 / 05279771001)
7. Reaction Buffer Concentrate (10X) (Cat. No. 950-300 / 05353955001)
8. LCS (Predilute) (Cat. No. 650-010 / 05264839001)
9. ULTRA LCS (Predilute) (Cat. No. 650-210 / 05424534001)
10. Cell Conditioning Solution (CC1) (Cat. No. 950-124 / 05279801001)
11. ULTRA Cell Conditioning Solution (ULTRA CC1) (Cat. No. 950-224 / 05424569001)
12. Hematoxylin II (Cat. No. 790-2208 / 05277965001)
13. Bluing Reagent (Cat. No. 760-2037 / 05266769001)
14. General purpose laboratory equipment
15. BenchMark IHC/ISH instrument

STORAGE AND STABILITY

Upon receipt and when not in use, store at 2-8°C. Do not freeze.

To ensure proper reagent delivery and the stability of the antibody, replace the dispenser cap after every use and immediately place the dispenser 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.

SPECIMEN PREPARATION

Routinely processed FFPE tissues are suitable for use with this primary antibody when used with VENTANA detection kits and BenchMark IHC/ISH instruments. The recommended tissue fixative is 10% neutral buffered formalin.[22] Slides should be stained immediately, as antigenicity of cut tissue sections may diminish over time.

It is recommended that positive and negative controls be run simultaneously with unknown specimens.

Approximately 4 µm thick sections should be cut and picked up on glass slides. The slides should be Superfrost Plus or equivalent. Studies indicate that air dried cut tissue and cell line sections stored at 2-8°C are stable for up to 6 months. Each laboratory should validate the cut slide stability for their own procedures and environmental storage conditions.

WARNINGS AND PRECAUTIONS

1. For in vitro diagnostic (IVD) use.
2. For professional use only.
3. CAUTION: In the United States, Federal law restricts this device to sale by or on the order of a physician. (Rx Only)
4. Do not use beyond the specified number of tests.
5. Positively charged slides may be susceptible to environmental stresses resulting in inappropriate staining. Ask your Roche representative for more information on how to use these types of slides.
6. The use of this product for selection of patients eligible for KADCYLA or PERJETA therapies may not be available in all geographies. Please consult your local Roche representative to confirm availability in specific locations.
7. Materials of human or animal origin should be handled as biohazardous materials and disposed of with proper precautions. In the event of exposure, the health directives of the responsible authorities should be followed.[23,24]
8. Avoid contact of reagents with eyes and mucous membranes. If reagents come in contact with sensitive areas, wash with copious amounts of water.
9. Avoid microbial contamination of reagents as it may cause incorrect results.
10. 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 criteria for a hazardous substance. Buildup of NaN3 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.[25] Systemic allergic reactions are possible in sensitive individuals.
11. For further information on the use of this device, refer to the BenchMark IHC/ISH instrument User Guide, and instructions for use of all necessary components located at dialog.roche.com.
12. Consult local and/or state authorities with regard to recommended method of disposal.
13. Product safety labeling primarily follows EU GHS guidance. Safety data sheet available for professional user on request.
14. To report suspected serious incidents related to this device, contact the local Roche representative and the competent authority of the Member State or Country in which the user is established.

STAINING PROCEDURE

VENTANA primary antibodies have been developed for use on BenchMark IHC/ISH instruments in combination with VENTANA detection kits and accessories. Refer to the tables below for recommended staining protocols.

This antibody has been optimized for specific incubation times but the user must validate results obtained with this reagent.

The parameters for the automated procedures can be displayed, printed and edited according to the procedure in the instrument User Guide. Refer to the appropriate VENTANA detection kit method sheet for more details regarding immunohistochemistry staining procedures.

For more details on the proper use of this device, refer to the inline dispenser method sheet associated with P/N 790-4493.

Table 1. Recommended staining protocol for VENTANA anti-HER2 (4B5) antibody with *i*VIEW DAB Detection Kit on BenchMark IHC/ISH instruments.

Procedure Type	Method		
	GX	XT	ULTRA
Deparaffinization	Selected	Selected	Selected
Cell Conditioning (Antigen Unmasking)	CC1, Standard	CC1, Standard	ULTRA CC1, Mild
Antibody (Primary)	32 minutes, 37°C	32 minutes, 37°C	24 minutes, 36°C
A/B Block (Biotin Blocking)	Not Selected	Selected	Selected
Counterstain	Hematoxylin II, 4 minutes		
Post Counterstain	Bluing, 4 minutes		

Table 2. Recommended staining protocol for VENTANA anti-HER2 (4B5) antibody with *ultra*View Universal DAB Detection Kit on BenchMark IHC/ISH instruments.

Procedure Type	Method		
	GX	XT	ULTRA or ULTRA PLUS
Deparaffinization	Selected	Selected	Selected

Procedure Type	Method		
	GX	XT	ULTRA or ULTRA PLUS
Cell Conditioning (Antigen Unmasking)	CC1, Mild	CC1, Mild	ULTRA CC1, Mild
Antibody (Primary)	16 minutes, 37 °C	16 minutes, 37 °C	12 minutes, 36 °C
ultraWash	Selected		
Counterstain	Hematoxylin II, 4 minutes		
Post Counterstain	Bluing, 4 minutes		

Due to variation in tissue fixation and processing, as well as general lab instrument and environmental conditions, it may be necessary to increase or decrease the primary antibody incubation, cell conditioning based on individual specimens, detection used, and reader preference. For further information on fixation variables, refer to "Immunohistochemistry Principles and Advances".[26]

Table 3 outlines the recommended staining procedures for breast and gastric carcinomas.

Table 3. Staining procedures on BenchMark IHC/ISH instruments.

	GX	XT	ULTRA or ULTRA PLUS
Breast Carcinoma	*i*VIEW DAB or *ultra*View DAB staining procedure*	*i*VIEW DAB or *ultra*View DAB staining procedure*	*i*VIEW DAB or *ultra*View DAB staining procedure *
Gastric Carcinoma	GX uVDAB HER2 4B5 Gastric (Table 2)	XT uVDAB HER2 4B5 Gastric (Table 2)	ULTRA uVDAB HER2 4B5 Gastric (Table 2)

* Breast Carcinoma Specimens stained on BenchMark IHC/ISH instruments are using the staining procedures for *i*VIEW DAB Detection Kit (Table 1) or *ultra*View Universal DAB Detection Kit (Table 2)

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. PATHWAY HER-2 4 in 1 Control Slides (P/N 781-2991) may be useful for a preliminary validation of the processing method used for staining slides with VENTANA anti-HER2 (4B5) antibody. These four cell line controls are characterized by in situ hybridization for gene copy number, Table 4. When processed and stained appropriately, the cell lines should stain as described in the PATHWAY HER-2 4 in 1 Control Slide method sheet. 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 4. 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 VENTANA anti-HER2 (4B5) antibody staining procedure performed. Optimal laboratory practice is to include a positive control section on the same slide as the test tissue. This helps identify any failures applying reagents to the slide. Tissue with weak positive staining is best suited for quality control. Control tissue may contain both positive and negative staining elements and serve as both the positive and negative control. Control tissue should be fresh autopsy, biopsy, surgical specimen, prepared or 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. 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 HER2 is strongly positive due to the nature of the pathology (overexpression).

Known positive tissue controls should be utilized only for monitoring performance of and reagents and instruments, not as an aid in determining specific diagnosis of test samples. If the positive tissue controls fail to demonstrate positive staining, results of the test specimen should be considered invalid.

An example of a positive control for VENTANA anti-HER2 (4B5) antibody is a known weak HER2 positive invasive breast carcinoma, or weakly positive gastric carcinoma specimen. The positive staining tissue components (membranous staining of neoplastic cells) are used to confirm that the antibody was applied and the instrument functioned properly.

Negative Tissue Control

The same slide used for the positive tissue control (ductal or lobular invasive breast carcinoma, or gastric 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 (false positive) with the primary antibody. Use a known negative tissue, fixed, processed and embedded in a manner identical to the patient sample(s).

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. Refer to the Troubleshooting section. 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,[27] or the CLSI Approved Guideline[28] 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 and gastric cancer tissues with known HER2 status are suitable for assay verification.

STAINING INTERPRETATION / EXPECTED RESULTS

The VENTANA automated immunostaining procedure causes a brown colored (DAB) reaction product to precipitate at the antigen sites localized by VENTANA anti-HER2 (4B5) antibody. 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. 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 VENTANA anti-HER2 (4B5) Antibody in Breast Carcinoma

Breast carcinomas that are considered positive for HER2 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. Scan complete tissue section to ensure scoring in well-preserved and well stained areas only. 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 40X 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 *Interpretation Guide for VENTANA anti-HER2/neu (4B5) Rabbit Monoclonal Primary Antibody Staining of Breast and Gastric Carcinoma* (P/N 1012716) for a more detailed description with photographs of staining with VENTANA anti-HER2 (4B5) antibody.

Table 5. Criteria for intensity and pattern of cell membrane staining with VENTANA anti-HER2 (4B5) antibody in breast carcinoma.

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 to moderate complete staining of the membrane, > 10% of cancer cells	2+	Equivocal*
Intense complete staining of the membrane, > 10% of cancer cells	3+	Positive

* Recommend reflex to ISH

Scoring Conventions for the Interpretation of VENTANA anti-HER2 (4B5) antibody in Gastric Carcinoma

Gastric carcinomas that are considered positive for HER2 protein overexpression must meet a threshold criteria for the intensity and pattern of membrane staining (2+ or greater on a scale of 0 to 3+), and for the percent positive tumor cells. Staining must localize to the cell membrane but need not be completely circumferential, as baso-lateral staining is regularly observed and should be considered for scoring. Staining of the cytoplasm and/or the nucleus may be present, but this staining is not included in the determination of positivity. In gastric carcinoma the percentage of positive tumor cells depends upon whether the sample is a biopsy specimen (≥ 5 cohesive cells) or resection specimen (≥ 10%).

In establishing the scoring guidelines for HER2 immunohistochemistry in gastric cancer note that while strong membranous staining is evidence of HER2 protein overexpression in neoplastic cells it need not be completely circumferential.[29]

Diffuse cytoplasmic staining with or without nuclear staining in gastric cancer has been reported.[30] Only membranous staining should be used in determination of HER2 protein expression in gastric cancer.

Immunohistochemical staining with the clone 4B5 can produce cytoplasmic and nuclear staining of normal gastric mucosa and more infrequently of neoplastic cells in gastric carcinoma and gastric/esophageal carcinoma. The nature of this cytoplasmic and nuclear staining is currently unknown. This staining pattern should not be confused with the discrete membranous staining, as that is indicative of HER2 positivity in neoplastic cells.

Refer to *Interpretation Guide for VENTANA anti-HER2/neu (4B5) Rabbit Monoclonal Primary Antibody Staining of Breast and Gastric Carcinoma* for a more detailed description with photomicrographs of staining with VENTANA anti-HER2 (4B5) antibody.

Table 6. Criteria for intensity and pattern of cell membrane staining with VENTANA anti-HER2 (4B5) antibody in gastric carcinoma.

Staining Pattern - Resection Specimen	Staining Pattern - Biopsy Specimen	Score (Report to requesting physician)	HER2 Staining Assessment
No reactivity or membranous reactivity in < 10% of tumor cells	No reactivity or membranous reactivity in any tumor cell	0	Negative
Faint/barely perceptible membranous reactivity in ≥ 10% of tumor cells; cells are reactive only in part of their membrane	Tumor cell cluster* with a faint/barely perceptible membranous reactivity irrespective of percentage of tumor cells stained	1+	Negative
Weak to moderate complete, basolateral or lateral membranous reactivity in ≥ 10% of tumor cells	Tumor cell cluster* with a weak to moderate complete, basolateral or lateral membranous reactivity irrespective of percentage of tumor cells stained	2+	Equivocal**
Strong complete, basolateral or lateral membranous reactivity in ≥ 10% of tumor cells	Tumor cell cluster* with a strong complete, basolateral or lateral membranous reactivity irrespective of percentage of tumor cells stained	3+	Positive

* ≥ 5 cohesive cells

** Recommend reflex to ISH

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 antibodies and reagents are provided 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.[31] 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.[32]
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.[33]
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

The antibody has been optimized as indicated in Table 1 and Table 2 for BenchMark IHC/ISH instruments 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".[26]

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.

Bone marrow was not tested for specificity. The user should determine appropriate staining in the above tissues prior to interpretation of staining information.

Immunohistochemical staining with clone 4B5 can produce cytoplasmic and nuclear staining of normal gastric mucosa and more infrequently of neoplastic cells in gastric carcinoma and gastric/esophageal carcinoma. The nature of this cytoplasmic and nuclear staining is currently unknown. This staining pattern should not be confused with the discrete membranous staining that is indicative of HER2 positivity in neoplastic cells.

All assays might not be registered on every instrument. Please contact your local Roche representative for more information.

PERFORMANCE CHARACTERISTICS

Staining tests for sensitivity, specificity, and precision were conducted and the results are listed below.

ANALYTICAL PERFORMANCE

Sensitivity and Specificity

VENTANA anti-HER2 (4B5) antibody sensitivity/specificity was determined by a study that showed no specific membrane staining for most normal tissues. Staining results are listed in Table 7. VENTANA anti-HER2 (4B5) antibody sensitivity and specificity was also determined by a study that showed no specific membrane staining in most neoplastic tissues. Staining results are listed in Table 8. Staining for sensitivity and specificity were performed using the *i*VIEW DAB Detection Kit protocol on a BenchMark XT instrument or the *ultra*View Universal DAB Detection Kit protocol on a BenchMark ULTRA instrument listed above.

Positive staining in tonsillar epithelium, esophageal epithelium, prostate, peripheral nerve, parathyroid, breast cancer, colon, and ovarian cancer are consistent with published literature regarding expression of HER2.

Table 7. Sensitivity/Specificity of VENTANA anti-HER2 (4B5) antibody was determined by testing FFPE normal tissues.

Tissue	# positive / total cases	Tissue	# positive / total cases
Cerebrum	0/6	Small intestine	0/6
Cerebellum	0/6	Colon	0/46
Adrenal gland	0/6	Liver	0/6
Ovary	0/6	Salivary gland	0/3
Pancreas	0/6	Tongue	0/3
Lymph Node	0/12	Kidney	0/6
Pituitary gland	0/5	Prostate	1/6
Testis	0/6	Bladder [b]	3/3
Thyroid	0/6	Rectum	0/6
Breast	0/14	Parathyroid gland [c]	4/6
Spleen	0/6	Endometrium	0/3
Tonsil [a]	3/6	Uterus	0/3
Thymus	0/5	Cervix	0/5
Bone marrow	0/3	Endocervix	0/1
Lung	0/6	Skeletal muscle	0/6
Heart	0/5	Skin	0/6
Pericardium	0/3	Nerve	2/6
Esophagus	1/6	Mesothelium	0/3
Stomach	0/11	N/A	N/A

[a] focal staining of surface epithelial cells

[b] membranous staining of superficial umbrella cells

[c] focal membrane staining

Table 8. Sensitivity/Specificity of VENTANA anti-HER2 (4B5) antibody was determined by testing a variety of FFPE neoplastic tissues.

Pathology	# positive / total cases
Glioblastoma (Cerebrum)	0/2

Pathology	# positive / total cases
Meningioma (Cerebrum)	0/1
Oligodendroglioma (Cerebrum)	0/1
Serous adenocarcinoma (Ovary)	0/2
Carcinoma, NOS (Not Otherwise Specified) (Ovary)	1/2
Neuroendocrine neoplasm (Pancreas)	0/1
Adenocarcinoma (Pancreas)	0/1
Carcinoma, NOS (Pancreas)	0/3
Seminoma (Testis)	0/1
Embryonal carcinoma (Testis)	0/1
Medullary carcinoma (Thyroid)	0/1
Papillary carcinoma (Thyroid)	0/1
Carcinoma, NOS (Thyroid)	0/3
Microinvasive ductal carcinoma (Breast)	2/2
Invasive ductal carcinoma (Breast)	44/99
Carcinoma, NOS (Breast)	1/4
Small cell carcinoma (Lung)	0/1
Squamous cell carcinoma (Lung)	0/1
Carcinoma, NOS (Lung)	0/2
Adenocarcinoma (Lung)	0/1
Squamous cell carcinoma (Esophagus)	0/1
Adenocarcinoma (Esophagus)	0/1
Mucinous adenocarcinoma (Stomach)	0/4
Adenocarcinoma (Stomach)	8/88
Signet-ring cell carcinoma (Stomach)	0/4
Carcinoma, NOS (Stomach)	0/3
Adenocarcinoma (Small Intestine)	0/1
Gastrointestinal stromal tumor (Small Intestine)	0/1
Adenocarcinoma (Colon)	0/32
Gastrointestinal Stromal Tumor (Colon)	0/1
Carcinoma, NOS (Colon)	1/3
Adenocarcinoma (Rectum)	1/5
Gastrointestinal Stromal Tumor (Rectum)	0/1
Melanoma (Rectum)	0/1
Hepatocellular carcinoma (Liver)	0/3
Hepatoblastoma (Liver)	0/1
Carcinoma, NOS (Liver)	0/3
Clear cell carcinoma (Kidney)	0/1

Pathology	# positive / total cases
Carcinoma, NOS (Kidney)	0/5
Adenocarcinoma (Prostate)	0/2
Carcinoma, NOS (Prostate)	0/3
Leiomyoma	0/3
Adenocarcinoma (Uterus)	0/1
Clear cell carcinoma (Uterus)	0/1
Squamous cell carcinoma (Cervix)	0/2
Embryonal rhabdomyosarcoma (Striated muscle)	0/1
Basal cell carcinoma (Skin)	0/1
Squamous cell carcinoma (Skin)	1/1
Neurofibroma (Lumbar)	0/1
Neuroblastoma (Retroperitoneum)	0/1
Mesothelioma (Peritoneum)	0/1
Pleomorphic rhabdomyosarcoma (Peritoneum)	0/1
Lymphoma, NOS	0/3
B-cell lymphoma, NOS (Spleen)	0/1
B-Cell lymphoma, NOS (Lymph node)	0/2
Hodgkin lymphoma (Lymph node)	0/1
Urothelial carcinoma (Bladder)	1/1
Leiomyosarcoma (Bladder)	0/1
Osteosarcoma (Bone)	0/1
Leiomyosarcoma (Smooth muscle)	0/1
Rectum adenocarcinoma (Metastatic)	0/1
Colon adenocarcinoma (Metastatic)	0/7
Colon mucinous adenocarcinoma (Metastatic)	0/1
Melanoma	0/2
Neuroendocrine neoplasm, NOS	0/2
Sarcoma, NOS	0/2
Undifferentiated carcinoma, NOS	0/1

Analytical Performance In Breast Cases

Performance characteristics on BenchMark ULTRA instrument using iVIEW DAB Detection Kit or *ultra*View Universal DAB Detection Kit

BenchMark ULTRA instrument 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 HER2 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 BenchMark ULTRA instrument using the recommended staining protocol and *ultra*View Universal DAB Detection Kit. Controls included the PATHWAY HER-2 4 in 1 Control 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.

The following tables are 3x3 presentations of results for each reader based on clinical score where 2+ and 3+ were separated.

Table 9. Site A vs. Site B Inter-laboratory Agreement Rates 3x3 Analysis–clone 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 10. Site A vs. Site C Inter-laboratory Agreement Rates 3x3 Analysis–clone 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 11. Site B vs. Site C Inter-laboratory Agreement Rates 3x3 Analysis–clone 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)	

BenchMark ULTRA instrument 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 instrument 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 clone 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.

Comparison study of BenchMark ULTRA instrument to BenchMark XT instrument

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 HER2 binning category (0, 1+, 2+, 3+)] were randomly distributed to two staining sites (140 cases to each site) for staining on a BenchMark XT instrument and a BenchMark ULTRA instrument using the respective recommended staining protocols and *ultra*View Universal DAB Detection Kit. Controls included the PATHWAY HER-2 4 in 1 Control 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 clone 4B5 antibody staining on the BenchMark ULTRA instrument versus BenchMark XT instrument based on clinical assessment (positive, negative) 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 clone 4B5 antibody staining on the BenchMark ULTRA instrument versus BenchMark XT instrument based on clinical assessment (positive, negative) 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 clone 4B5 staining using BenchMark ULTRA instrument versus BenchMark XT instrument based on 2x2 analysis of clinical assessment (positive, negative) 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 the tables below:

Table 12. BenchMark ULTRA instrument vs. BenchMark XT instrument 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 13. BenchMark ULTRA instrument vs. BenchMark XT instrument 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 14. BenchMark ULTRA instrument vs. BenchMark XT instrument 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)	

Inter-pathologist reproducibility of instrument 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%).

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 clone 4B5 on BenchMark ULTRA instrument. A single staining laboratory and three reading sites in the United States participated in the detection comparison study. For clone 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 the tables below

Table 15. Reader A, *i*VIEW DAB Detection Kit vs. *ultra*View Universal DAB Detection Kit Agreement Rates 3x3 Analysis–clone 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 16. Reader B, *i*VIEW DAB Detection Kit vs. *ultra*View Universal DAB Detection Kit Agreement Rates 3x3 Analysis–clone 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 17. Reader C, *i*VIEW DAB Detection Kit vs. *ultra*View Universal DAB Detection Kit Agreement Rates 3x3 Analysis–clone 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)	

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.

Inter-Laboratory and Inter-Reader Reproducibility Studies on BenchMark ULTRA PLUS Instrument Using ***ultra***View Universal DAB Detection Kit

Three laboratories, from separate institutions in the United States, participated in the inter-laboratory reproducibility study. Cut slides of 28 FFPE invasive breast carcinoma cases [7 each from HER2 binning categories (0, 1+, 2+, 3+)] and a pair of control slides (one PATHWAY HER-2 4 in 1 Control Slide and one control breast carcinoma tissue case) for each of 10 staining runs were distributed to each study site for staining on a BenchMark ULTRA PLUS instrument using the recommended staining protocol and *ultra*View Universal DAB Detection Kit. Controls included the PATHWAY HER-2 4 in 1 Control Slides, a control breast carcinoma tissue case and a second slide of each test 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.

The reproducibility of HER2 status (positive or negative) was evaluated for all evaluable case slides using the case-level modal HER2 status as the reference for each case. For the purpose of calculating agreement rates, a HER2 score of 0 or 1+ was considered negative and a HER2 score of 2+ or 3+ was considered positive. The modal HER2 status

for each case was determined as the most frequently observed reader result (positive or negative) for the given case. All observations obtained by pooling all sites, readers, days, and cases for the all evaluable case slide population were compared against the modal HER2 status results. The PPA, NPA and OPA for the pooled analysis were 97.9% (411/420), 97.6% (410/420), and 97.7% (821/840), respectively. Reproducibility of HER2 status was also evaluated as the APA, ANA, and OPA rates for all possible pairwise comparisons between sites and for all site comparisons combined. For Inter-site reproducibility, APA, ANA, and OPA were 95.9%, 95.9%, and 95.9%, respectively. For Between-Reader reproducibility, the results for APA, ANA, and OPA were 95.5%, 95.5%, and 95.5%. For Between-Day reproducibility, APA, ANA, and OPA were 97.0%, 97.0%, and 97.0%. Two-sided 95% CIs were calculated using the percentile bootstrap method.

Tables 18-21 are presentations of these results.

Table 18. Pooled Agreement of the HER2 Status with Case-Level Modal Status for Breast Carcinoma Cases Stained with the anti-HER2 (4B5) antibody on the BenchMark ULTRA PLUS.

HER2 Status	BenchMark ULTRA PLUS Modal Status		Total
	Positive	Negative	
Positive	411	10	421
Negative	9	410	419
Total	420	420	840
	n/N	% (95% CI)	
Positive Percent Agreement	411/420	97.9 (95.7, 99.5)	
Negative Percent Agreement	410/420	97.6 (94.3, 100.0)	
Overall percent agreement	821/840	97.7 (96.0, 99.3)	

Table 19. Site pooled agreement rates for the pairwise comparisons of HER2 status for Breast Carcinoma Cases Stained with the anti-HER2 (4B5) antibody on the BenchMark ULTRA PLUS.

HER2 Status from Site i	HER2 Status from Site j		Total
	Positive	Negative	
Positive	4037	163	4200
Negative	183	4017	4200
Total	4220	4180	8400
	n/N	% (95% CI)	
Average Positive Agreement	8074/8420	95.9 (92.8, 98.6)	
Average Negative Agreement	8034/8380	95.9 (92.5, 98.7)	
Overall percent agreement	8054/8400	95.9 (92.7, 98.6)	

Note: Site i and j are the sites (Site A, Site B, and Site C) used in the study. The pooled agreement rate is calculated by pooling all possible comparison between all sites (Site A vs. B, A vs. C, and B vs. C).

Table 20. Between-Reader pooled agreement rates for the pairwise comparisons of HER2 status for Breast Carcinoma Cases Stained with the anti-HER2 (4B5) antibody on the BenchMark ULTRA PLUS.

HER2 Status from Reader 1	HER2 Status from Reader 2		Total
	Positive	Negative	
Positive	201	9	210
Negative	10	200	210
Total	211	209	420
	n/N	% (95% CI)	
Average Positive Agreement	402/421	95.5 (92.0, 98.6)	
Average Negative Agreement	400/419	95.5 (91.6, 98.6)	
Overall percent agreement	401/420	95.5 (91.9, 98.6)	

Note: Reader 1 and 2 are the readers from the same study site (Site A, Site B, or Site C) used in the study. The pooled agreement rate is calculated by pooling between reader agreements within a site across all study sites.

Table 21. Between-Day pooled agreement rates for the pairwise comparisons of HER2 status for Breast Carcinoma Cases Stained with the anti-HER2 (4B5) antibody on the BenchMark ULTRA PLUS.

HER2 Status from Day i	HER2 Status from Day j		Total
	Positive	Negative	
Positive	817	15	832
Negative	35	813	848
Total	852	828	1680
	n/N	% (95% CI)	
Average Positive Agreement	1634/1684	97.0 (95.0, 98.9)	
Average Negative Agreement	1626/1676	97.0 (94.8, 98.9)	
Overall percent agreement	1630/1680	97.0 (95.0, 98.9)	

Note: Day i and j are the days (Day 1 to Day 5) from the study staining days. The pooled agreement rate is calculated by pooling all possible comparison between any two days within each reader from each site.

Comparison Study of BenchMark ULTRA PLUS Instrument to BenchMark ULTRA Instrument

Three laboratories, from separate institutions in the United States, participated in a concordance study between the BenchMark ULTRA PLUS instrument versus the BenchMark ULTRA instrument. Cut slides of 122 FFPE invasive breast carcinoma cases from each HER-2 binning category (0, 1+, 2+, 3+) were stained by Ventana on a BenchMark ULTRA instrument using the recommended staining protocol and *ultra*View Universal DAB Detection Kit. Controls included one PATHWAY HER-2 4 in 1 Control Slide and one positive control breast carcinoma tissue case per staining run. Additionally, a second slide of each test case was stained with negative Ig reagent. Cut slides from the same cases were randomized and equally distributed (40-41 cases/per site) across study sites for staining on a BenchMark ULTRA PLUS instrument using the recommended staining protocol and *ultra*View Universal DAB Detection Kit. Controls included one PATHWAY HER-2 4 in 1 Control Slide and one positive control breast carcinoma tissue case per staining run. Additionally, a second slide of each test case was stained with negative Ig reagent. Pathologists, blinded to case status, evaluated the slides stained on one BenchMark IHC/ISH instrument and provided a clinical score (i.e., 0, 1+, 2+, 3+). After a 2 week washout period, pathologists evaluated the slides stained on the second BenchMark IHC/ISH instrument. The results were analyzed by Ventana. A HER2 score of IHC 0+ or 1+ was considered negative, and a HER2 score of IHC 2+ or 3+ was considered positive. The OPA, PPA and NPA rates were 91.0% (333/366), 93.3% (154/165), and 89.1% (179/201), respectively. The two-sided 95% CIs were calculated using the percentile bootstrap method. Background and morphology acceptability rates for all cases were 100% for both instruments. Table 22 is the presentation of these results.

Table 22. Pooled Agreement of the HER2 Status for Breast Carcinoma Cases Stained with the anti-HER2 (4B5) antibody on the BenchMark ULTRA PLUS vs BenchMark ULTRA Instruments

BenchMark ULTRA PLUS HER2 Status	BenchMark ULTRA HER2 Status		Total
	Positive	Negative	
Positive	154	22	176
Negative	11	179	190
Total	165	201	366
	n/N	% (95% CI)	
Positive Percent Agreement	154/165	93.3 (89.5, 96.9)	
Negative Percent Agreement	179/201	89.1 (85.1, 93.0)	
Overall percent agreement	333/366	91.0 (88.5, 93.7)	

Analytical Performance In Gastric Cases

BenchMark ULTRA and BenchMark XT instrument precision studies:

Inter-run repeatability on the BenchMark XT instrument was performed in five runs conducted over a 5 day (non-consecutive) period. Five slides containing three gastric tissue cases with scores of 0, 1+, 2+, and 3+ HER2 expression demonstrated 100% agreement within the positive/negative value for each tissue.

Intra-run repeatability on the BenchMark XT instrument was performed on 28 slides containing three gastric tissue cases with scores of 0, 1+, 2+, and 3+ HER2 expression. All cases scored equivalently within the positive/negative value for each tissue type.

Intra-platform repeatability was performed across three BenchMark XT instruments. In these runs all 30 slides from each of two different multi tissue blocks containing three gastric tissue cases with scores of 0, 1+, 2+, and 3+ HER2 expression scored equivalently within the positive/negative value for each tissue type.

Intra-platform repeatability was tested across three BenchMark ULTRA instruments. In these runs all 15 slides from one multi-tissue block scored equivalently within the positive/negative value for each tissue type.

Inter-platform repeatability was tested across three BenchMark XT and three BenchMark ULTRA instruments. In these runs all 30 slides from one multi-tissue block scored equivalently within the positive/negative value for each tissue type.

Comparison of *i*VIEW DAB Detection Kit and ***ultra***View Universal DAB Detection Kit using Gastric Cases

Clone 4B5 was used to conduct detection kit comparison testing across two instruments (BenchMark XT instrument and BenchMark ULTRA instrument), using *i*VIEW DAB Detection Kit and *ultra*View Universal DAB Detection Kit. Two hundred and ten tissue cases were used as part of the testing. The stained slides were evaluated for positive/negative clinical scoring.

The morphology and background acceptability rates were 100% for both detection kits and instruments. Direct comparisons for positive and negative clinical assessment between detection kits, for each instrument are presented in the following tables.

Table 23. Clinical assessment for *ultra*View Universal DAB Detection Kit versus *i*VIEW DAB Detection Kit on the BenchMark XT instrument.

ultraView Universal DAB Detection Kit	*i*VIEW DAB Detection Kit		
	Positive	Negative	Total
Positive	21	0	21
Negative	0	189	189
Total	21	189	210
	n/N	%	
Positive percent agreement	21/21	100	
Negative percent agreement	189/189	100	
Overall percent agreement	210/210	100	

Table 24. Clinical assessment comparison on the BenchMark XT and BenchMark ULTRA instruments using *ultra*View Universal DAB Detection Kit.

BenchMark XT instrument with ***ultra***View Universal DAB Detection Kit	BenchMark ULTRA instrument with ***ultra***View Universal DAB Detection Kit		
	Positive	Negative	Total
Positive	20	1	21
Negative	0	189	189
Total	20	190	210
	n/N	% (95% CI)	
Positive percent agreement	20/20	100 (83.9-100)	
Negative percent agreement	189/190	99.5 (97.1-99.9)	
Overall percent agreement	209/210	99.5 (97.4-99.9)	

Inter-laboratory reproducibility of clone 4B5 in Gastric Carcinoma:

The study was conducted at three test sites. Specimens were selected for inclusion in the study based on clone 4B5 IHC clinical score, such that there were an approximately equal number of positive (3+) and negative (0, 1+) cases. Additionally, up to four cases of 2+ qualified gastric cancer cases were studied.

The three sites each used a BenchMark XT instrument and a BenchMark ULTRA instrument to conduct four staining runs per instrument. Cases were randomized for staining using a stratified randomization procedure that assigned cases such that each run contained cases representing all scoring categories for HER2 in gastric cancer. The runs on each instrument at each site contained the same cases. At each site, one slide from each case was stained with clone 4B5 and another slide from the same case was stained with CONFIRM Negative Control Rabbit Ig on the BenchMark ULTRA instrument. A second pair of slides from the same case was similarly stained on the BenchMark XT instrument at each site. Case slides were scored by one qualified reader at each site blinded to previously determined IHC clinical scores for each specimen.

The overall agreement for all evaluable cases was 100% for all three site-to-site comparisons on both the BenchMark ULTRA instrument and the BenchMark XT instrument. The overall agreement between the BenchMark ULTRA instrument and BenchMark XT instrument for evaluable cases was 100% at each of the three sites. Background and morphology acceptability rates for all cases were 100% for both instruments at Sites A and C and > 95% for both instruments at Site B. See tables below.

Table 25. Overall clinical assessment agreement between sites: gastric carcinoma, all evaluable cases.

BenchMark ULTRA instrument	Percent Overall Agreement
Site A vs Site B: n/N (%) (95% CI)	30/30 (100%) (88.6 – 100)
Site A vs Site C: n/N (%) (95% CI)	30/30 (100%) (88.6 – 100)
Site B vs Site C: n/N (%) (95% CI)	30/30 (100%) (88.6 – 100)
BenchMark XT instrument	**Percent Overall Agreement**
Site A vs Site B: n/N (%) (95% CI)	31/31 (100%) (89.0 – 100.0)
Site A vs Site C: n/N (%) (95% CI)	31/31 (100%) (89.0 – 100.0)
Site B vs Site C: n/N (%) (95% CI)	31/31 (100%) (89.0 – 100.0)

Table 26. Overall clinical assessment agreement between platforms: gastric carcinoma all evaluable cases.

BenchMark ULTRA instrument vs BenchMark XT instrument	Percent Overall Agreement
Site A: n/N (%) (95% CI)	40/40 (100%) (91.2 – 100)
Site B: n/N (%) (95% CI)	34/34 (100%) (89.8 – 100)
Site C: n/N (%) (95% CI)	32/32 (100%) (89.3 – 100)

Table 27. Background staining and morphology acceptability rates: gastric carcinoma all cases.

BenchMark ULTRA instrument	Site A	Site B	Site C
Morphology Acceptability Rates	44/44 (100%)	43/44 (97.7%)	44/44 (100%)
Background Acceptability Rates	44/44 (100%)	42/44 (95.5%)	44/44 (100%)
BenchMark XT instrument	**Site A**	**Site B**	**Site C**
Morphology Acceptability Rates	44/44 (100%)	43/44 (97.7%)	44/44 (100%)
Background Acceptability Rates	44/44 (100%)	43/44 (97.7%)	44/44 (100%)

Comparison study of BenchMark instrument and BenchMark GX instrument to BenchMark XT instrument: Gastric Carcinoma

Cut slides of 3 TMAs containing FFPE gastric carcinoma cases [approximately 50 cases per TMA] were stained on a BenchMark XT instrument, BenchMark instrument and BenchMark GX instrument using the respective recommended staining protocols for *ultra*View Universal DAB Detection Kit and *i*VIEW DAB Detection Kit. Controls included the PATHWAY HER-2 4 in 1 Control Slides and a second slide of each TMA stained with negative Ig reagent. Stained slides were scored by one reader (pathologist).

The overall agreement rates (and lower bound of the two-sided 95% confidence intervals) for clone 4B5 antibody staining based on clinical assessment (positive, negative) were as follows: BenchMark instrument versus BenchMark XT instrument with *ultra*View Universal DAB Detection Kit 98.0% (94.2-99.3), BenchMark GX instrument versus BenchMark XT instrument with *ultra*View Universal DAB Detection Kit 97.4% (93.6-99.0), BenchMark instrument versus BenchMark XT instrument with *i*VIEW DAB Detection Kit 96.6% (92.7-98.4), BenchMark GX instrument versus BenchMark XT instrument with *i*VIEW DAB Detection Kit 95.9% (91.8-98.0).

The positive agreement rates (and lower bound of the two-sided 95% confidence intervals) for clone 4B5 antibody staining based on clinical assessment (positive, negative) were as follows: BenchMark instrument versus BenchMark XT instrument with *ultra*View Universal DAB Detection Kit 91.7% (64.4-98.5), BenchMark GX instrument versus BenchMark XT instrument with *ultra*View Universal DAB Detection Kit 78.6% (52.4-92.4), BenchMark instrument versus BenchMark XT instrument with *i*VIEW DAB Detection Kit 80.0% (54.8-93.0), BenchMark GX instrument versus BenchMark XT instrument with *i*VIEW DAB Detection Kit 73.3% (48.0-89.1).

The negative agreement rates (and lower bound of the two-sided 95% confidence intervals) for clone 4B5 staining based on clinical assessment (positive, negative) were as follows: BenchMark instrument versus BenchMark XT instrument with *ultra*View Universal DAB Detection Kit 98.5% (94.8-99.6), BenchMark GX instrument versus BenchMark XT instrument with *ultra*View Universal DAB Detection Kit 99.3% (96.1-99.9), BenchMark instrument versus BenchMark XT instrument with *i*VIEW DAB Detection Kit 98.1% (94.6-99.4), BenchMark GX instrument versus BenchMark XT instrument with *i*VIEW DAB Detection Kit 98.1% (94.5-99.3). The 2x2 presentation of the agreement rates for each comparison based on clinical assessment (positive, negative) are shown in the tables below.

Table 28. BenchMark instrument vs. BenchMark XT instrument Inter-Platform Agreement Rates with *ultra*View Universal DAB Detection Kit 2x2 Analysis: gastric carcinoma.

Clone 4B5 with ***ultra***View Universal DAB Detection Kit			
	BenchMark XT instrument		
BenchMark instrument	Positive	Negative	Total
Positive	11	2	13
Negative	1	133	134
Total	12	135	147
	n/N	% (95% CI)	
Overall percent agreement	144/147	98.0% (94.2-99.3)	
Positive percent agreement	11/12	91.7% (64.6-98.5)	
Negative percent agreement	133/135	98.5% (94.8-99.6)	

Table 29. BenchMark GX instrument vs. BenchMark XT instrument Inter-Platform Agreement Rates with *ultra*View Universal DAB Detection Kit 2x2 Analysis: gastric carcinoma.

Clone 4B5 with ***ultra***View Universal DAB Detection Kit			
	BenchMark XT instrument		
BenchMark GX instrument	Positive	Negative	Total
Positive	11	1	12
Negative	3	140	143
Total	14	141	155
	n/N	% (95% CI)	
Overall percent agreement	151/155	97.4% (93.6-99.0)	
Positive percent agreement	11/14	78.6% (52.4-92.4)	
Negative percent agreement	140/141	99.3% (96.1-99.9)	

Table 30. BenchMark instrument vs. BenchMark XT instrument Inter-Platform Agreement Rates with *i*VIEW DAB Detection Kit, 2x2 Analysis: gastric carcinoma.

Clone 4B5 with *i*VIEW DAB Detection Kit			
	BenchMark XT instrument		
BenchMark instrument	Positive	Negative	Total
Positive	12	3	15
Negative	3	156	159
Total	15	159	174
	n/N	% (95% CI)	
Overall percent agreement	168/174	96.6% (92.7-98.4)	
Positive percent agreement	12/15	80.0% (54.8-93.0)	
Negative percent agreement	156/159	98.1% (94.6-99.4)	

Table 31. BenchMark GX instrument vs. BenchMark XT instrument Inter-Platform Agreement Rates with *i*VIEW DAB Detection Kit, 2x2 Analysis: gastric carcinoma.

Clone 4B5 with *i*VIEW DAB Detection Kit			
	BenchMark XT instrument		
BenchMark GX instrument	Positive	Negative	Total
Positive	11	3	14
Negative	4	154	158
Total	15	157	172
	n/N	% (95% CI)	
Overall percent agreement	165/172	95.9% (91.8-98.0)	
Positive percent agreement	11/15	73.3% (48.0-89.1)	
Negative percent agreement	154/157	98.1% (94.5-99.3)	

BenchMark ULTRA PLUS Instrument Precision Studies

Intra-run repeatability on the BenchMark ULTRA PLUS instrument was performed on 120 slides across 24 gastric tissue cases with scores of 0, 1+, 2+, and 3+ HER2 expression. All cases scored equivalently within the positive/negative value for each tissue type.

Inter-run repeatability on the BenchMark ULTRA PLUS instrument was performed in five runs conducted over a 5 day (non-consecutive) period yielding 240 slides across 24 gastric tissue cases with scores of 0, 1+, 2+, and 3+ HER2 expression. All cases scored equivalently within the positive/negative value for each tissue type.

Intra-platform repeatability was performed across three BenchMark ULTRA PLUS instruments. These runs yielded 144 slides from across 24 gastric tissue cases with scores of 0, 1+, 2+, and 3+ HER2 expression. All cases scored equivalently within the positive/negative value for each tissue type.

Concordance of Results Between BenchMark ULTRA PLUS and BenchMark ULTRA Instruments

Cut slides of 94 FFPE gastric carcinoma cases were stained on a BenchMark ULTRA PLUS instrument and BenchMark ULTRA instrument using the respective recommended staining protocols for *ultra*View Universal DAB Detection Kit. Controls included a HER2 positive gastric carcinoma case for each staining run and a second test slide stained with negative Ig reagent. Stained slides were scored by one reader (pathologist).

The overall agreement rates for anti-HER2 (4B5) antibody staining based on status (positive, negative) was 98.9%. Positive agreement rates and negative agreement rates were 100.0% and 98.4%, respectively. Two-sided 95% confidence intervals were calculated using the Wilson score method. Background and morphology acceptability rates for all cases were 100% for both instruments.

CLINICAL PERFORMANCE

Comparison studies of clone 4B5 rabbit monoclonal antibody to PATHWAY anti-HER2 (CB11) Mouse Monoclonal Antibody in Breast Cancer

A method comparison study was conducted to examine the correlation of clone 4B5 to PATHWAY anti-HER2 (CB11) Mouse Monoclonal Antibody (PATHWAY anti-HER2 (CB11) antibody) and PathVysion HER2 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 = 178, Cohort 2: n = 144) using known breast cancer cases stained with PATHWAY anti-HER2 (CB11) antibody and clone 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 HER2 scoring.[34,35,36] A total of 322 cases were evaluated. The slides stained with PATHWAY anti-HER2 (CB11) antibody were processed and stained according to the manufacturer's instructions specified in the PATHWAY anti-HER2 (CB11) antibody method sheet. There was an average of approximately one year between staining and reading of the PATHWAY anti-HER2 (CB11) antibody stained slides. Since scores from one of the six readers was outside of the confidence interval (CI), data from the two cohorts are presented as follows:

Inter-pathologist Reproducibility of Comparison Studies Specimens

Table 32. Cohort 1: Consensus IHC scores of three pathologists.

Clone 4B5 Score	PATHWAY anti-HER2 (CB11) antibody 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% CI = 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% CI = 97.5% - 100%).
- Negative percent agreement is 53/75 = 70.7% (95% CI = 58.5% - 80.1%).

Overall agreement is (29+24+2+13+53)/143 = 84.7% (95% CI = 78.2% - 90.0).

Table 33. Cohort 2: Consensus IHC scores of three pathologists.

Clone 4B5 Score	PATHWAY anti-HER2 (CB11) antibody 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% CI = 80.1%, 93.1%).

Cohort 2: Performance characteristics for 2 x 2 Presentation (HER2 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% CI = 85.2% - 96.9%).
- Negative percent agreement is 80/85 = 94.1% (95% CI = 86.8% - 98.1%).

Overall agreement is (72+12+1+1+80)/178 = 93.3% (95% CI = 88.5% - 96.4%).

Table 34. Cohort 1: Consensus PATHWAY anti-HER2 (CB11) antibody IHC scores of three pathologists compared to FISH.

PATHWAY anti-HER2 (CB11) antibody Score	FISH Result		
	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 PATHWAY anti-HER2 (CB11) antibody IHC 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% CI = 63.8% - 83.2%).
- Negative percent agreement is 53/58 = 91.4% (95% CI = 80.9% - 97.1%).

Overall agreement is (32+32+53)/144 = 81.2% (95% CI = 73.9% - 87.2%).

Table 35. Cohort 1: Consensus clone 4B5 IHC scores of three pathologists compared to FISH.

Clone 4B5 Score	FISH Result		
	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 Clone 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% CI = 87.9% - 96.3%).
- Negative percent agreement is 47/58 = 81.0% (95% CI = 73.4% - 86.0%).

Overall agreement is (55+25+47)/144 = 88.2% (95% CI = 82.1% - 92.2%).

Table 36. Cohort 2: Consensus PATHWAY anti-HER2 (CB11) antibody IHC scores of three pathologists compared to FISH.

PATHWAY HER2 (CB11) antibody Score	FISH Result		
	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 PATHWAY anti-HER2 (CB11) antibody IHC 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% CI = 85.0% - 96.7%).
- Negative percent agreement is 77/85 = 90.6% (95% CI = 83.9% - 96.3%).

Overall agreement is (72+13+77)/178 = 91.0% (95% CI = 86.5% - 94.9%).

Table 37. Cohort 2: Consensus clone 4B5 IHC scores of three pathologists compared to FISH.

Clone 4B5 Score	FISH Result		
	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 Clone 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% CI = 82.5% - 95.1%).
- Negative percent agreement is 77/85 = 90.6% (95% CI = 84.0% - 96.4%).

Overall agreement is (72+11+77)/178 = 90.0% (95% CI = 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.

Table 38. Cohort 1: Clone 4B5 scoring for the three pathologists.

HER2 Score	Clone 4B5 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 39. Cohort 1: PATHWAY anti-HER2 (CB11) antibody IHC scoring for the three pathologists.

HER2 Score	PATHWAY anti-HER2 (CB11) antibody 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 40. Cohort 2: Clone 4B5 scoring for the three pathologists.

HER2 Score	Clone 4B5 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 41. Cohort 2: PATHWAY anti-HER2 (CB11) antibody IHC scoring for the three pathologists.

HER2 Score	PATHWAY anti-HER2 (CB11) antibody 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 42. Ranges of 2X2* agreements for the three pathologists.

	Overall Percent Agreement	Positive Percent Agreement	Negative Percent Agreement
Clone 4B5 vs. PATHWAY anti-HER2 (CB11) antibody			
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%
Clone 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%
PATHWAY anti-HER2 (CB11) antibody 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: This study demonstrated that there is clinically significant concordance (overall agreement between positive/negative results) between the clone 4B5 assay and PATHWAY anti-HER2 (CB11) assay thereby demonstrating that the HER2 clone 4B5 is an acceptable alternative to the CB11 antibody for use as an aid in the assessment of breast cancer patients for whom trastuzumab (Herceptin) therapy is being considered. This study demonstrated that HER2 expression results obtained from the clone 4B5 IHC assay are comparable to HER2 gene status results determined by FISH analysis.

Comparison of Clone 4B5 to HercepTest in Human Gastric Cancer:

A blinded, external study was conducted to compare the staining performance of the clone 4B5 on the BenchMark XT instrument to that of the Dako HercepTest. Two cohorts of samples were studied, (1) newly constructed tissue microarrays (TMAs) containing 248 gastric cancer cases (six cases were later found to be duplicates and were removed), and (2) a subset of 183 clinical trial samples from the Trastuzumab for Gastric Cancer (ToGA) Trial that investigated HER2 status and clinical outcome in patients treated with Herceptin (trastuzumab). The laboratory stained the cases with clone 4B5 and HercepTest. A total of 431 cases were evaluable by both assays were included in the comparison. A pathologist scored the cases on a scale of 0/1+, 2+, and 3+. Positive cases consist of scores of 2+ and 3+, while negative cases are 0 and 1+. Table 43 includes agreement rates between clone 4B5 and HercepTest, for both cohorts studied.

Table 43. Agreement data for clone 4B5 (IHC) vs. HercepTest in gastric carcinoma.

Tissue Source	Overall Percent Agreement (95% CI)	Positive Percent Agreement (95% CI)	Negative Percent Agreement (95% CI)
TMA[a] & ToGA[b]	91.0 (87.7-93.4)	82.1 (70.2-90.0)	92.4 (89.1-94.8)
n	362 / 398	46 / 56	316 / 342

IHC results were considered antibody positive (2+ and 3+) and negative (0+ and 1+).

[a] TMA:tissue micro array samples

[b] ToGA:clinical trial specimens from the ToGA trial

Method Comparison to Enrollment Assay of PERJETA (pertuzumab) and KADCYLA (trastuzumab emtansine) Studies in Breast Carcinoma

Equivalency to enrollment assays for cohorts from PERJETA and KADCYLA studies was determined by staining of trial specimens with VENTANA anti-HER2 (4B5) antibody assay. 2753 specimens evaluated for a PERJETA trial and 99 specimens evaluated for a KADCYLA trial were stained with VENTANA anti-HER2 (4B5) antibody. Agreement rates (PPA, NPA and OPA) were determined. The 95% CI (2-sided 95% confidence interval) was calculated using the score method.

Table 44. Agreement of the Clone 4B5 and Dako Assays on HER2 Status for all HER2 evaluable subjects. IHC evaluable subjects have a HER2 status of Positive or Negative determined by both the Clone 4B5 and the enrollment IHC assay.

		Dako HER2 Status [a,b]		
Study	Clone 4B5 Score [b]	Positive	Negative	Total
PERJETA and KADCYLA	3+	2380	15	2395
	2+	140	122	262
	0/1+	38	135	173
	Total	2558	272	2830
	Positive Percent Agreement n/N (%) (95% CI)	2380/2558 (93.0) (92.0-94.0)		
	Negative Percent Agreement n/N (%) (95% CI)	257/272 (94.5) (91.1-96.6)		
	Overall Percent Agreement n/N (%) (95% CI)	2637/2830 (93.2) (92.2-94.1)		

[a] Positive = IHC Positive and/or ISH Amplified. Negative = IHC Negative and not ISH Amplified or ISH Non-Amplified and not IHC Positive.

[b] IHC: Positive = 3+; Negative = 0, 1+, or 2+.

Table 45. Agreement of Clone 4B5 and Dako Assays on IHC Status for all IHC evaluable subjects. IHC evaluable subjects have a HER2 status of Positive or Negative determined by both Clone 4B5 and the enrollment IHC assay.

		Dako HercepTest Status [a]		
Study	Clone 4B5 Status [a]	Positive	Negative	Total
PERJETA and KADCYLA	Positive	2330	65	2395
	Negative	21	414	435
	Total	2351	479	2830
	Positive Percent Agreement n/N (%) (95% CI)	2330/2351 (99.1) (98.6-99.4)		
	Negative Percent Agreement n/N (%) (95% CI)	414/479 (86.4) (83.1-89.2)		
	Overall Percent Agreement n/N (%) (95% CI)	2744/2830 (97.0) (96.3-97.5)		
PERJETA	Positive	2267	63	2330
	Negative	10	399	409
	Total	2277	462	2739
	Positive Percent Agreement n/N (%) (95% CI)	2267/2277 (99.6) (99.2-99.8)		
	Negative Percent Agreement n/N (%) (95% CI)	399/462 (86.4) (82.9-89.2)		
	Overall Percent Agreement n/N (%) (95% CI)	2666/2739 (97.3) (96.7-97.9)		
KADCYLA	Positive	63	2	65
	Negative	11	15	26
	Total	74	17	91
	Positive Percent Agreement n/N (%) (95% CI)	63/74 (85.1) (75.3-91.5)		
	Negative Percent Agreement n/N (%) (95% CI)	15/17 (88.2) (65.7-96.7)		
	Overall Percent Agreement n/N (%) (95% CI)	78/91 (85.7) (77.1-91.5)		

[a] Positive = 3+; Negative = 0, 1+, or 2+.

Table 46. Agreement of Clone 4B5 and Dako Assays on IHC Score for all IHC evaluable subjects. IHC evaluable subjects have a HER2 status of Positive or Negative determined by both the Clone 4B5 and the enrollment IHC assay.

		Dako HercepTest Score			
Study	Clone 4B5 Score	3+	2+	0/1+	Total
PERJETA and KADCYLA	3+	2330	64	1	2395
	2+	12	235	15	262
	0/1+	9	26	138	173
	Total	2351	325	154	2830
	Overall Percent Agreement n/N (%) (95% CI)	2703/2830 (95.5) (94.7-96.2)			
PERJETA	3+	2267	62	1	2330
	2+	9	226	13	248
	0/1+	1	24	136	161
	Total	2277	312	150	2739
	Overall Percent Agreement n/N (%) (95% CI)	2629/2739 (96.0) (95.2-96.7)			
KADCYLA	3+	63	2	0	65
	2+	3	9	2	14
	0/1+	8	2	2	12
	Total	74	13	4	91
	Overall Percent Agreement n/N (%) (95% CI)	74/91 (81.3) (72.1-88.0)			

Table 47. Clone 4B5 Staining Acceptability. IHC Tested Subjects. IHC staining is considered acceptable if a valid IHC score (0, 1+, 2+, or 3+) could be determined. Reasons for unacceptable staining include unacceptable negative control, tissue loss, insufficient tumor, unacceptable background, and unacceptable morphology.

Parameter	PERJETA	KADCYLA	PERJETA and KADCYLA
Number of Initial IHC Tests	2753	99	2852
Initial Staining Acceptability n/N (%) (95% CI)	2708/2753 (98.4) (97.8, 98.8)	92/99 (92.9) (86.1, 96.5)	2800/2852 (98.2) (97.6, 98.6)
Number of Repeat IHC Tests	40	0	40
Final Staining Acceptability n/N (%) (95% CI)	2746/2753 (99.7) (99.5, 99.9)	92/99 (92.9) (86.1, 96.5)	2838/2852 (99.5) (99.2, 99.7)

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 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. If nuclear and cytoplasmic staining are present in normal mucosa in close proximity to the tumor area in gastric carcinoma, and confuses interpretation of membrane staining, the case can be tested by ISH.
7. For corrective action, refer to the Staining Procedure section, the instrument User Guide or contact your local support representative.

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NOTE: A point (period/stop) is always used in this document as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.

The summary of safety and performance can be found here:

https://ec.europa.eu/tools/eudamed

Symbols

Ventana uses the following symbols and signs in addition to those listed in the ISO 15223-1 standard (for USA: see dialog.roche.com for definition of symbols used):

GTIN	Global Trade Item Number
UDI	Unique Device Identification
	Indicates the entity importing the medical device into the European Union

REVISION HISTORY

Rev	Updates
D	Updates made to Intended Use, Summary and Explanation, Clinical Significance, Principle of the Procedure, Material Provided, Materials Required But Not Provided, Specimen Preparation, Warnings and Precautions, Quality Control Procedures, Positive Tissue Control, Staining Interpretation/Expected Results, Analytical Performance, References. Addition of BenchMark ULTRA PLUS.

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