VENTANA®

Roche

VENTANA HER2 (4B5) Rabbit Monoclonal Primary Antibody RxDx

REF 790-7167

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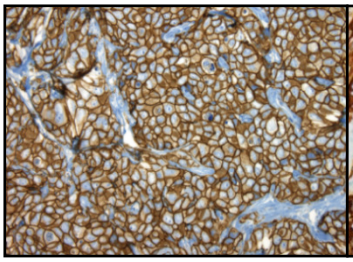

Figure 1. VENTANA HER2 (4B5) Rabbit Monoclonal Primary Antibody RxDx staining in breast carcinoma.

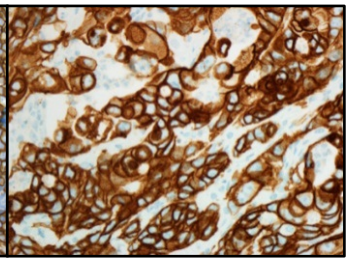

Figure 2. VENTANA HER2 (4B5) Rabbit Monoclonal Primary Antibody RxDx 3+ staining in gastric carcinoma.

INTENDED USE

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

This IHC device is indicated as a companion diagnostic for identifying breast cancer patients who are eligible for treatment with trastuzumab (IHC 3+ or IHC 2+/ISH amplified), pertuzumab (IHC 3+ or IHC 2+/ISH amplified), trastuzumab emtansine (IHC 3+ or IHC 2+/ISH amplified), or trastuzumab deruxtecan (IHC 1+ or IHC 2+/ISH non-amplified). Additionally, this IHC device is indicated as a companion diagnostic for identifying gastric cancer patients for whom trastuzumab treatment is being considered (IHC 3+ or IHC 2+/ISH amplified).

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 HER2 (4B5) Rabbit Monoclonal Primary Antibody RxDx (VENTANA HER2 (4B5) Assay) contains a rabbit monoclonal antibody (clone 4B5) directed against the internal domain of human epidermal growth factor receptor 2 (HER2). HER2 was cloned and characterized.[1] Clone 4B5 has been shown to react with a 185 kDa 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 for the HER2 protein (185 kDa).[1] Immunohistochemistry (IHC) experiments with transfected cell lines (HEK293) have shown that clone 4B5 stains cells transfected with HER2 and cells transfected with HER4 though no staining of cells transfected with HER1 or HER3 was observed and Western blot data with recombinant HER4 protein also indicated that clone 4B5 recognizes a HER4 epitope.[3] Despite this, HER2 (4B5) has not been observed to cross-react with HER4 in IHC staining of formalin-fixed, paraffin-embedded (FFPE) breast tissue.[4]

HER2 is a transmembrane receptor tyrosine kinase which is structurally similar to epidermal growth factor receptor.[5,6] Gene amplification and the corresponding overexpression of HER2 has been found in a variety of tumors, including breast carcinomas.[5,6,7] Protein overexpression, due to amplification of the HER2 gene, is the primary driver of HER2 mediated tumorigenesis.[5] Gene amplification typically results in a significant increase in HER2 receptors at the cell membrane.[5,6] Overexpression of HER2 enhances signal transduction and upregulates proliferation and differentiation, ultimately causing tumor formation.[5,6]

A spectrum of HER2 protein expression has been observed in the absence of gene amplification.[8] Several factors have been proposed to explain intermediate levels of HER2 protein expression in breast cancer cases in the absence of gene amplification, including crosstalk between the HER2 and estrogen receptor signaling pathways.[8,9] HER2 protein expression that is not considered overexpression may be classified as HER2-low expression.[8,10,11]

HER2 protein overexpression and/or gene amplification occurs in gastric and gastro-esophageal junction adenocarcinoma.[12,13,14] A wide range of HER2 overexpression frequency has been reported across published studies. However, one of the largest screening datasets which included 3803 patients with gastric and gastroesophageal junction adenocarcinoma reported that 22 percent of patients tested positive for HER2 protein overexpression or gene amplification.[15]

CLINICAL SIGNIFICANCE

Breast cancer is the most commonly diagnosed cancer in women worldwide.[16] Early detection and appropriate treatment selection can significantly affect overall survival.[7,17] Approximately 15 to 30 percent of invasive ductal cancers of the breast are positive for HER2.[7,10] Almost all cases of Paget's disease of breast and up to 90 percent of cases of ductal carcinoma in situ of comedo type are positive.[18,19] HER2-positive status has defined a subgroup of breast cancer patients who benefit from HER2-targeted therapy for more than 20 years.[7,17] The HER2-positive population has historically been defined as those patients that demonstrate HER2 protein overexpression assessed by immunohisto-chemistry (IHC) based on a semi-quantitative IHC scoring system (0, 1+, 2+ and 3+) and/or gene amplification assessed by in-situ hybridization (ISH).[17] HER2-positivity has been strongly correlated with protein overexpression (IHC score of 3+). In cases with borderline overexpression (IHC score 2+, equivocal) a confirmatory reflex test to assess gene amplification may be required per the established HER2 assessment algorithm.[17]

On-market therapeutic drugs, including Herceptin (trastuzumab), PERJETA (pertuzumab) and KADCYLA (ado-trastuzumab emtansine / trastuzumab emtansine) have demonstrated clinical benefit in HER2-positive breast cancer patients by arresting, and in some cases reversing the growth of their cancer.[20-24] Trastuzumab and pertuzumab are humanized monoclonal antibodies that bind to HER2 protein on the cell surface and disrupt HER2-mediated signal transduction.[20,24,25] Trastuzumab emtansine is an antibody-drug conjugate composed of trastuzumab and the cytotoxic agent DM1 conjugated through a non-cleavable linker.[23] Only patients with HER2-positive breast cancer (IHC score 3+ or 2+ with a confirmed HER2 amplified status) should benefit from treatment with trastuzumab (Herceptin), pertuzumab (PERJETA) or trastuzumab emtansine (KADCYLA).[16,18]

Approximately 40-50 percent of breast cancer patients have tumors that do not demonstrate amplification of the HER2 gene and do not overexpress the receptor; however, low levels of HER2 expression are detected.[8,10] HER2-low expressing cases (IHC score 1+ or 2+ (with a confirmed HER2 non-amplified status)) are typically considered HER2-negative and excluded from HER2-targeted treatment options.[8] Recently, benefit has been observed with the anti-HER2 treatment trastuzumab deruxtecan (ENHERTU®) in breast cancer patients with low levels of HER2 expression.[26,27] Trastuzumab deruxtecan is an antibody-drug conjugate that contains a HER2 targeting monoclonal antibody (trastuzumab) base, a cleavable linker and a cell membrane permeable exatecan derivative (a topoisomerase I inhibitor payload).[10]

In vitro diagnostics for the evaluation of HER2 status in breast cancer patients are important to aid the clinician in the determination of therapy with trastuzumab (Herceptin), pertuzumab (PERJETA), trastuzumab emtansine (KADCYLA) or trastuzumab deruxtecan (ENHERTU®).[17] 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 trastuzumab (Herceptin), pertuzumab (PERJETA), trastuzumab emtansine (KADCYLA) or trastuzumab deruxtecan (ENHERTU®) are being considered.

Gastric cancer is the fifth most common cancer and a leading cause of cancer-related death globally.[13] Surgery is the most common treatment for gastric cancer.[14,28] However, most gastric cancer cases are detected at an advanced stage and the surgery is often difficult to perform.[14,28] Chemotherapy is used for treating advanced gastric cancer even though the survival of these patients is very low.[14,28] The HER2 targeted therapy trastuzumab is a mainstay in the management of invasive breast cancer and has therapeutic value in the management of gastric cancer patients overexpressing the receptor.[12,14] Demonstration of *HER2* gene amplification and/or protein overexpression is essential for selecting patients for trastuzumab therapy.[12,14] Clinical studies have shown

that breast or gastric cancer patients with high HER2 protein overexpression and/or gene amplification benefit most from trastuzumab.[12,21] The IHC-based detection of HER2 protein expression is used as an aid in the assessment of gastric cancer patients for whom trastuzumab (Herceptin) treatment is being considered.

PRINCIPLE OF THE PROCEDURE

VENTANA HER2 (4B5) Assay contains a rabbit monoclonal antibody, which binds to HER2 in FFPE tissue sections. The specific antibody can be localized using 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 carcinoma). In addition to staining with VENTANA HER2 (4B5) Assay, 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 a BenchMark IHC/ISH instrument. This negative tissue control should be stained with VENTANA HER2 (4B5) RxDx to ensure that the antigen enhancement and other pretreatment procedures did not create false positive staining.

MATERIAL PROVIDED

VENTANA HER2 (4B5) Assay contains sufficient reagent for 50 tests.

One 5 mL dispenser of VENTANA HER2 (4B5) Assay 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 HER2 (4B5) Assay contains 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. CONFIRM Negative Control Rabbit Ig (Cat. No. 760-1029 / 05266238001)
4. *ultra*View DAB Detection Kit (Cat. No. 760-500 / 05269806001)
5. EZ Prep Concentrate (10X) (Cat. No. 950-102 / 05279771001)
6. Reaction Buffer Concentrate (10X) (Cat. No. 950-300 / 05353955001)
7. LCS (Predilute) (Cat. No. 650-010 / 05264839001)
8. ULTRA LCS (Predilute) (Cat. No. 650-210 / 05424534001)
9. Cell Conditioning Solution (CC1) (Cat. No. 950-124 / 05279801001)
10. ULTRA Cell Conditioning Solution (ULTRA CC1) (Cat. No. 950-224 / 05424569001)
11. Hematoxylin II (Cat. No. 790-2208 / 05277965001)
12. Bluing Reagent (Cat. No. 760-2037 / 05266769001)
13. General purpose laboratory equipment
14. 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.[29] 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 a minimum of 45 days. 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. Do not use beyond the specified number of tests.
4. 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.
5. 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.[30,31]
6. Avoid contact of reagents with eyes and mucous membranes. If reagents come in contact with sensitive areas, wash with copious amounts of water.
7. Avoid microbial contamination of reagents as it may cause incorrect results.
8. 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.[32] Systemic allergic reactions are possible in sensitive individuals.
9. 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 navifyportal.roche.com.
10. Consult local and/or state authorities with regard to recommended method of disposal.
11. Product safety labeling primarily follows EU GHS guidance. Safety data sheet available for professional user on request.
12. 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 *ultra*View DAB Detection Kit method sheet for more details regarding IHC staining procedures.

For more details on the proper use of this device, refer to the inline dispenser method sheet associated with P/N 790-7167.

NOTE: Staining Procedures are summarized in Table 1 (For HER2-positivity & HER2-low assessment in breast cancer cases), Table 2 (For HER2-positivity assessment only in breast cancer cases) and Table 3 (For HER2-positivity assessment in gastric cancer cases).The recommended staining protocol steps, including cell conditioning and antibody incubation are the same for all HER2 breast cancer screening; however, the staining procedures have varying levels of user alteration available. When screening patients for potential HER2-low assessment, the staining procedure in Table 1 must be utilized. If the intent may include to report out HER2-low status (defined as IHC score 1+ or 2+ (with a confirmed HER2 non-amplified status) then the staining procedure listed in Table 1 must be used. If the intent is only to report out HER2-positivity status (defined as IHC score 3+ or 2+ with a confirmed HER2 amplified status), the procedures in Table 1 or Table 2 may be used. To ensure most comprehensive utilization of results for either HER2-low or HER2-positivity status in breast cancer cases, it is strongly recommended to use the staining procedure listed in Table 1.

Staining Procedure for All HER2 Assessment Including Potential HER2-low Assessment in Breast Specimens

The staining protocol and procedure listed in this section and Table 1 is appropriate for use in all HER2 screening of breast carcinoma cases, and must be used when assessing patient samples for potential HER2-low therapy. Deviating from the recommended staining protocol may produce unacceptable HER2-stained samples with a changed HER2 Score, particularly in cases with low HER2 expression (IHC 1+). Decreasing or increasing cell conditioning times in particular are likely to produce HER2-stained samples with altered HER2 scores, which may result in inappropriate treatment decisions for patients. The staining procedure listed in Table 1 does not allow alteration of the cell conditioning or antibody incubation time to assist in mitigating this risk, as this is the only staining protocol validated for use in assessment of potential HER2-low patient samples.

Table 1. Staining Protocol for VENTANA HER2 (4B5) Assay for all HER2 assessment in breast specimens including potential HER2-low assessment on a BenchMark IHC/ISH instrument.

Procedure Type	Method		
	BenchMark GX	BenchMark XT	BenchMark ULTRA or BenchMark ULTRA PLUS
Staining Procedure	GX VENTANA HER2 4B5	XT VENTANA HER2 4B5	ULTRA VENTANA HER2 4B5
Antibody (Primary)*	VENTANA HER2 4B5 Ab-16 min. or CONFIRM Neg Ctl Rbt Ig-16 min.	VENTANA HER2 4B5 Ab-16 min. or CONFIRM Neg Ctl Rbt Ig-16 min.	VENTANA HER2 4B5 Ab-12 min. or CONFIRM Neg Ctl Rbt Ig-12 min.
Counterstain	Hematoxylin II, 4 minutes		
Post Counterstain	Bluing, 4 minutes		

* This is a pre-programmed condition and is not a selectable step for the user.

Alternative Staining Procedures Only Applicable for Potential HER2-positivity Assessment in Breast and Gastric Specimens

The staining procedures and protocols listed in Table 2 and Table 3 are intended for use in assessing HER2-positivity (defined as protein overexpression, IHC score 3+ or 2+ (with a confirmed HER2 amplified status) and not in assessing HER2-low expression (defined as IHC score 1+ or 2+ (with a confirmed HER2 non-amplified status). The recommended cell conditioning and antibody incubation times detailed in Table 2 and Table 3 are not locked and may be altered by the user. Potential reasons for altering parameters include variation in tissue fixation and processing (deviation from recommended fixation or processing of samples), general lab instrument and environmental conditions, and reader preference. For further information on fixation variables, refer to "Immunohistochemistry Principles and Advances."[33] Users who deviate from recommended staining protocols are responsible for interpretation and validation of patient results.

Table 2. Recommended staining protocol for VENTANA HER2 (4B5) Assay with *ultra*View DAB Detection Kit in breast specimens on a BenchMark IHC/ISH instrument.

Procedure Type	Method		
	BenchMark GX	BenchMark XT	BenchMark ULTRA or BenchMark ULTRA PLUS
Staining Procedure	*ultra*View DAB staining procedure	*ultra*View DAB staining procedure	*ultra*View DAB staining procedure
Deparaffinization	Selected	Selected	Selected
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		

Table 3. Recommended staining protocol for VENTANA HER2 (4B5) Assay with *ultra*View DAB Detection Kit in gastric specimens on a BenchMark IHC/ISH instrument.

Procedure Type	Method		
	BenchMark GX	Benchmark XT	BenchMark ULTRA
Staining Procedure	*ultra*View DAB staining procedure	*ultra*View DAB staining procedure	*ultra*View DAB staining procedure
Deparaffinization	Selected	Selected	Selected
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		

QUALITY CONTROL PROCEDURES

Cell Line 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 instrument or processing method used for staining slides with VENTANA HER2 (4B5) Assay. 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 HER2 (4B5) Assay 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 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 HER2 (4B5) Assay 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.

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,[34] or the CLSI Approved Guideline[35] 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 HER2 (4B5) Assay. 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

The following tables provide staining criteria. Refer to VENTANA HER2 (4B5) Rabbit Monoclonal Primary Antibody RxDx Interpretation Guide for Breast and Gastric Cancer (P/N 1021957EN) for a more detailed description with images of staining with VENTANA HER2 (4B5) Assay.

Table 5. Criteria for Intensity and Pattern of Cell Membrane Staining with VENTANA HER2 (4B5) Assay in Breast Carcinoma when using the staining procedure in Table 1.

Staining pattern	HER2 (4B5) Score (Report to treating physician)	Using Staining Procedure in Table 1: Recommended Reporting Status	Clinical Application
No membrane staining is observed or, Faint, partial staining of the membrane in 10% or less of the cancer cells	0	HER2 negative	None
Faint, partial staining of the membrane in greater than 10% of the cancer cells	1+	HER2-low expression	ENHERTU® (trastuzumab deruxtecan)
Weak to moderate complete staining of the membrane in greater than 10% of the cancer cells	2+* Reflex test: HER2 non-amplified	HER2-low expression	
	2+* Reflex test: HER2 amplified	HER2 positive / overexpression	HERCEPTIN (trastuzumab), PERJETA (pertuzumab), KADCYLA (trastuzumab emtansine)
Intense complete staining of the membrane in greater than 10% of the cancer cells	3+	HER2 positive / overexpression	

* Recommend reflex test to assess gene amplification per ASCO/CAP guidance

Table 6. Criteria for Intensity and Pattern of Cell Membrane Staining with VENTANA HER2 (4B5) RxDx Assay in Breast Carcinoma when using the staining procedure in Table 2.

Staining pattern	HER2 (4B5) Score (Report to treating physician)	Using Staining Procedure in Table 2: Recommended Reporting Status	Clinical Application
No membrane staining is observed or, Faint, partial staining of the membrane in 10% or less of the cancer cells	0	HER2 negative	None
Faint, partial staining of the membrane in greater than 10% of the cancer cells	1+	HER2 negative	
Weak to moderate complete staining of the membrane in greater than 10% of the cancer cells	2+* Reflex test: HER2 non-amplified	HER2 negative	
	2+* Reflex test: HER2 amplified	HER2 positive / overexpression	HERCEPTIN (trastuzumab), PERJETA (pertuzumab), KADCYLA (trastuzumab emtansine)
Intense complete staining of the membrane in greater than 10% of the cancer cells	3+	HER2 positive / overexpression	

* Recommend reflex test to assess gene amplification per ASCO/CAP guidance

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.[36]

Diffuse cytoplasmic staining with or without nuclear staining in gastric cancer has been reported.[37] 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 gastroesophageal junction 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 HER2 (4B5) Rabbit Monoclonal Primary Antibody RxDx Interpretation Guide for Breast and Gastric Cancer (P/N 1021957EN) for a more detailed description with photomicrographs of staining with VENTANA HER2 (4B5) Assay.

Table 7. Criteria for Intensity and Pattern of Cell Membrane Staining with VENTANA HER2 (4B5) Assay in gastric carcinoma using the staining procedure in Table 3.

Staining Pattern - Resection Specimen	Staining Pattern -Biopsy Specimen	Score (Report to treating physician)	HER2 Staining Assessment	Clinical Application
No reactivity or membranous reactivity in < 10% of tumor cells	No reactivity or membranous reactivity in any tumor cell	0	HER2 negative	None
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+	HER2 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+** Reflex test: HER2 non-amplified	HER2 negative	
		2+** Reflex test: HER2 amplified	HER2 positive / overexpression	HERCEPTIN (trastuzumab)
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+	HER2 positive / overexpression	

* ≥ 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.[38] 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.[39]
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.[40]
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. This antibody has been optimized as indicated in Table 1 and Table 2 for BenchMark instruments and detection chemistries. Deviating from the recommended staining protocols in Table 1 and Table 2 may produce unacceptable Negative Reagent Control (NRC) samples, and VENTANA HER2 (4B5) Assay stained samples with a changed HER2 Score. Increased antibody incubation time is likely to produce unacceptable staining in the NRC, which would prevent the VENTANA HER2 (4B5) Assay sample from being evaluated. Decreased and increased cell conditioning times are likely to produce VENTANA HER2 (4B5) Assay samples with changed HER2 scores which may cause inappropriate treatment decisions for patients. For further information on fixation variables, refer to "Immunohistochemistry Principles and Advances".[33]
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. Slides should be stained promptly, as antigenicity of cut tissue sections may diminish over time and may be compromised due to environmental factors during extended storage. Air dried slides should be desiccated and stored at 2-8°C. Studies support a minimum of 45 days of antigen stability on unstained slides. Laboratories should validate expiration dating within their own environment if dating beyond 45 days is desired.
4. Bone marrow was not tested for specificity. The user should determine appropriate staining in the above tissues prior to interpretation of staining information.
5. 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 gastroesophageal junction 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.

6. All assays might not be registered on every instrument. Please contact your local Roche representative for more information.
7. Changes in HER2 status have been reported to occur with metastatic progression or after neoadjuvant chemotherapy. Based on these observations it may be warranted to obtain a fresh sample for determining HER2 status at the time of treatment instead of relying upon historical HER2 status.[41]

PERFORMANCE CHARACTERISTICS

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

ANALYTICAL PERFORMANCE

Sensitivity and Specificity

VENTANA HER2 (4B5) Assay sensitivity/specificity was determined by a study that showed no specific membrane staining for most normal tissues. Staining results are listed in Table 8. VENTANA HER2 (4B5) Assay 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 9. 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.

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

Table 8. Sensitivity/Specificity of VENTANA HER2 (4B5) Assay 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 9. Sensitivity/Specificity of VENTANA HER2 (4B5) Assay was determined by testing a variety of FFPE neoplastic tissues.

Pathology	# positive / total cases
Glioblastoma (Cerebrum)	0/2
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)	44/98
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
Carcinoma, NOS (Kidney)	0/5
Adenocarcinoma (Prostate)	0/2
Carcinoma, NOS (Prostate)	0/3

Pathology	# positive / total cases
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 HER2-low Breast Cancer

Repeatability and Intermediate Precision for HER2-low on BenchMark ULTRA

Twenty-four breast carcinoma cases spanning the HER2 IHC staining range were included in the repeatability and intermediate precision study. The study design verified staining precision on breast carcinoma tissues stained with VENTANA HER2 (4B5) Assay.

Three lots of VENTANA HER2 (4B5) Assay (between-antibody lot)

Three lots of *ultra*View DAB IHC Detection Kits (between-detection kit lot)

Across three days (between-day)

Three BenchMark ULTRA instruments (between-instrument)

Across all intermediate precision conditions (Within-run)

Each sample was assigned one mode based on the samples aggregated per test condition for between-antibody lot, between-detection kit lot, between-instrument and between-day. For within-run condition, each sample was compared within its duplicate samples per test run. All slides were blinded and randomized, and then evaluated using the Criteria for Intensity and Pattern of Cell Membrane Staining with VENTANA HER2 (4B5) Assay staining (Table 5). Results are summarized in Table 10.

Table 10. Repeatability and intermediate precision of VENTANA HER2 (4B5) Assay on breast cancer tissues with HER2-low scoring

Repeatability/ Precision	Agreement			
	Type	n/N	%	95% CI
Between-Antibody Lots	PPA	96/96	100.0	(96.2, 100.0)
	NPA	48/48	100.0	(92.6, 100.0)
	OPA	144/144	100.0	(97.4, 100.0)
Between-Detection Kits	PPA	93/96	96.9	(92.2, 100.0)
	NPA	48/48	100.0	(92.6, 100.0)
	OPA	141/144	97.9	(94.4, 100.0)
Between-Instruments (BenchMark ULTRA)	PPA	95/96	99.0	(96.7, 100.0)
	NPA	48/48	100.0	(92.6, 100.0)
	OPA	143/144	99.3	(97.9, 100.0)
Between-Day	PPA	94/96	97.9	(93.3, 100.0)
	NPA	48/48	100.0	(92.6, 100.0)
	OPA	142/144	98.6	(95.8 100.0)
Within-Run	PPA	142/144	98.6	(96.5, 100.0)
	NPA	72/72	100.0	(94.9, 100.0)
	OPA	214/216	99.1	(97.7, 100.0)

Note: Positive Percent Agreement (PPA), Negative Percent Agreement (NPA), Overall Percent Agreement (OPA).

Note: Two-sided 95% confidence interval (CI) was calculated using the percentile bootstrap method from 2000 bootstrap samples. CIs for 100% PPA, NPA and OPA were calculated using Wilson score method.

Note: For the purposes of study analysis, HER2 scores 0 and 3+ were grouped together as negative cases because they were ineligible for HER2-low therapy per the clinical trial design, and HER2 scores of 1+ and 2+ were grouped together as positive cases as they were eligible or potentially eligible for HER2-low targeted therapy per the trial design.

Comparison Study of BenchMark ULTRA to Benchmark XT and Benchmark GX for HER2-low

Ten breast carcinoma cases spanning the HER2 IHC staining range were included in the intermediate precision study. The study design verified staining precision on breast carcinoma tissues stained with VENTANA HER2 (4B5) Assay across multiple instruments and multiple platforms.

Each sample was assigned one mode based on the samples aggregated per test condition. Each sample was compared within its duplicate samples per test run. All slides were blinded and randomized, and then evaluated using the criteria for intensity and pattern of cell membrane staining with VENTANA HER2 (4B5) Assay staining (Table 5). Results are summarized in Table 11.

Table 11. Repeatability and intermediate precision of VENTANA HER2 (4B5) Assay on breast cancer tissues with HER2-low scoring

Repeatability/ Precision	Agreement			
	Type	n/N	%	95% CI
Between Platforms (ULTRA/GX/XT)	PPA	90/90	100.0	(95.9, 100.0)
	NPA	90/90	100.0	(95.9, 100.0)
	OPA	180/180	100.0	(97.9, 100.0)
Between-Instrument (BenchMark ULTRA)	PPA	30/30	100.0	(88.6, 100.0)
	NPA	30/30	100.0	(88.6, 100.0)
	OPA	60/60	100.0	(94.0, 100.0)
Between-Instrument (BenchMark GX)	PPA	30/30	100.0	(88.6, 100.0)
	NPA	30/30	100.0	(88.6, 100.0)
	OPA	60/60	100.0	(94.0, 100.0)
Between-Instrument (BenchMark XT)	PPA	30/30	100.0	(88.6, 100.0)
	NPA	30/30	100.0	(88.6, 100.0)
	OPA	60/60	100.0	(94.0, 100.0)

Note: Positive Percent Agreement (PPA), Negative Percent Agreement (NPA), Overall Percent Agreement (OPA).

Note: Two-sided 95% confidence interval (CI) was calculated using the percentile bootstrap method from 2000 bootstrap samples. CIs for 100% PPA, NPA and OPA were calculated using Wilson score method.

Note: For the purposes of study analysis, HER2 scores 0 and 3+ were grouped together as negative cases because they were ineligible for HER2-low therapy per the clinical trial design, and HER2 scores of 1+ and 2+ were grouped together as positive cases as they were eligible or potentially eligible for HER2-low targeted therapy per the trial design.

Reader Precision for HER2-low on BenchMark ULTRA

Between-Reader and Within-Reader precision was assessed by evaluating concordance of HER2-low status between three readers and within three individual readers. The study included 100 breast carcinoma cases spanning the HER2 IHC staining range. Samples were blinded and randomized prior to evaluation for HER2-low status per Pattern of Cell Membrane Staining with VENTANA HER2 (4B5) Assay staining (Table 5). Readers scored all specimens twice, with a minimum of two weeks between reads. The agreement for between-reader and within-reader precision are summarized in Table 12.

Table 12. Within and Between-Reader Precision of the VENTANA HER2 (4B5) Assay with HER2-low scoring

Precision	Agreement			
	Type	n/N	%	95% CI
Within-Reader	APA	312/333	93.7	(90.9, 96.4)
	ANA	246/267	92.1	(88.0, 95.6)
	OPA	279/300	93.0	(90.0, 96.0)
Between-Reader	APA	300/332	90.4	(85.8, 94.3)
	ANA	236/268	88.1	(82.1, 93.0)
	OPA	268/300	89.3	(84.7, 94.0)

Note: Average Positive Agreement (APA), Average Negative Agreement (ANA), Overall Percent Agreement (OPA).

Note: Two-sided 95% confidence interval (CI) was calculated using the percentile bootstrap method from 2000 bootstrap samples.

Note: For the purposes of study analysis, HER2 scores 0 and 3+ were grouped together as negative cases because they were ineligible for HER2-low therapy per the clinical trial design, and HER2 scores of 1+ and 2+ were grouped together as positive cases as they were eligible or potentially eligible for HER2-low targeted therapy per the trial design.

Inter-laboratory Reproducibility Study for HER2-low on BenchMark ULTRA

An Inter-Laboratory Reproducibility Study of the VENTANA HER2 (4B5) Assay was completed to demonstrate reproducibility of the assay to determine HER2-low status of breast carcinoma cases. The study included 28 de-identified, archival, FFPE breast carcinoma tissue specimens run across three BenchMark ULTRA instruments on each of five non-consecutive days over 20 days at three external laboratories. The specimens represented the range of staining of the VENTANA HER2 (4B5) Assay.

Each set of 5 stained slides per sample per staining day was randomized and evaluated by a total of 6 readers (2 readers/ site) for a HER2-low status. The HER2-low status results for all readers, sites and days for the samples were combined and analyzed versus the reader modes for the same samples to determine the overall reproducibility of HER2-low status. The summary of the agreement rates across all evaluable observations, using the sample-level reader modes for HER2-low status as the reference can be found in Table 13.

Table 13. Inter-Laboratory Reproducibility for overall agreement rates for VENTANA HER2 (4B5) Assay with HER2-low scoring

Inter-Laboratory Reproducibility	Agreement			
	Type	n/N	%	95% CI
Overall	PPA	407/416	97.8	(96.2, 99.3)
	NPA	416/418	99.5	(98.8, 100.0)
	OPA	823/834	98.7	(97.7, 99.4)
Within-Site	PPA	407/416	97.8	(96.2, 99.3)
	NPA	416/418	99.5	(98.8, 100.0)
	OPA	823/834	98.7	(97.7, 99.4)
Within-Reader	PPA	407/416	97.8	(96.2, 99.3)
	NPA	416/418	99.5	(98.8, 100.0)
	OPA	823/834	98.7	(97.7, 99.4)

Note: Positive Percent Agreement (PPA), Negative Percent Agreement (NPA), Overall Percent Agreement (OPA).

Note: Two-sided 95% CIs were calculated using the percentile bootstrap method with 2000 replicates.

Note: For the purposes of study analysis, HER2 scores 0and 3+ were grouped together as negative cases because they were ineligible for HER2-low therapy per the clinical trial design, and HER2 scores of 1+ and 2+ were grouped together as positive cases as they were eligible or potentially eligible for HER2-low targeted therapy per the trial design.

In addition, pairwise comparisons were made Between-Site, Between-Reader and Between-Day for HER2-low status. A summary of the results can be found in Table 14. The data indicate assay reproducibility across 5 days, 3 sites, and 6 readers.

Table 14. Inter-Laboratory Reproducibility Pairwise Agreement Rates for the VENTANA HER2 (4B5) Assay with HER2-low scoring

Inter-Laboratory Reproducibility	Agreement			
	Type	n/N	%	95% CI
Between-Site	APA	7884/8102	97.3	(95.4, 98.8)
	ANA	8240/8458	97.4	(95.7, 98.8)
	OPA	8062/8280	97.4	(95.5, 98.8)
Between-Reader	APA	398/409	97.3	(95.4, 98.8)
	ANA	414/425	97.4	(95.6, 98.8)
	OPA	406/417	97.4	(95.5, 98.8)
Between-Day	APA	1580/1620	97.5	(95.9, 98.9)
	ANA	1652/1692	97.6	(96.2, 98.9)
	OPA	1616/1656	97.6	(96.1, 98.9)

Note: Average Positive Agreement (APA), Average Negative Agreement (ANA), Overall Percent Agreement (OPA)

Note: Two-sided 95% CIs were calculated using the percentile bootstrap method with 2000 replicates

Note: For the purposes of study analysis, HER2 scores 0 and 3+ were grouped together as negative cases because they were ineligible for HER2-low therapy per the clinical trial design, and HER2 scores of 1+ and 2+ were grouped together as positive cases as they were eligible or potentially eligible for HER2-low targeted therapy per the trial design.

Analytical Performance in HER2-low Breast Cases

Concordance Between BenchMark ULTRA and BenchMark ULTRA PLUS Instruments for HER2-low

Three laboratories participated in a concordance study to evaluate performance equivalence between the BenchMark ULTRA instrument and the BenchMark ULTRA PLUS instrument. For HER2-low statistical analysis, 160 (80 positive and 80 negative, including 16 borderline cases) of the cases were pre-selected for analysis prior to pathologist reads. Tissue slides from all cases were stained with a negative reagent control and VENTANA HER2 (4B5) Assay at an internal Roche laboratory on a BenchMark ULTRA instrument using the recommended staining protocol. Unstained tissue slides from all cases were randomized and equally distributed for staining on a BenchMark ULTRA PLUS instrument using the recommended VENTANA HER2 (4B5) staining protocol. Blinded to case status, one reader per site read the BenchMark ULTRA PLUS stained slides from their site and determined the HER2 (4B5) status. The results were analyzed by Roche. The results are summarized in Table 15.

Table 15. Pooled Agreement of HER2-low status for Cases Stained with VENTANA HER2 (4B5) Assay on the BenchMark ULTRA versus BenchMark ULTRA PLUS Instrument.

BenchMark ULTRA PLUS	BenchMark ULTRA				
	Roche Reader= Positive, External Reader= Positive	Roche Reader= Positive, External Reader= Negative	Roche Reader= Negative, External Reader= Positive	Roche Reader= Negative, External Reader= Negative	Total
Positive	272	13	25	11	321
Negative	8	9	2	298	317
Total	280	22	27	309	638
Percent Positive % (n/N)	97.1 (272/280)	59.1 (13/22)	92.6 (25/27)	3.6 (11/309)	N/A

	n/N	% (95% CI)
PPA	285/302	94.4 (91.6, 96.8)
NPA	300/336	89.3 (84.3, 94.4)
OPA	585/638	91.7 (88.5, 94.6)

Note: PPA = Positive Percent Agreement; NPA = Negative Percent Agreement; OPA = Overall Percent Agreement.

Note: Two-sided 95% CI calculated using the percentile bootstrap method with 2000 replicates stratified by IHC qualification score bin.

Note: The pooled agreement included all cases and ULTRA PLUS readers.

Note: For the purposes of study analysis, HER2 scores 0 and 3+ were grouped together as negative cases because they were ineligible for the clinical trial investigating HER2-low breast cancer. HER2 scores of 1+ and 2+ were grouped together as positive cases as they were eligible or potentially eligible for the clinical trial.

Inter-Laboratory Reproducibility Study- BenchMark ULTRA PLUS for HER2-low

An Inter-Laboratory Reproducibility Study of the VENTANA HER2 (4B5) Assay was completed to demonstrate reproducibility of the assay to determine HER2-low status of breast carcinoma cases.

The study included 28 de-identified, archival, FFPE breast carcinoma tissue specimens run across three BenchMark ULTRA PLUS instruments on each of five non-consecutive days over 20 days at three external laboratories. The specimens represented the range of staining of the VENTANA HER2 (4B5) Assay.

Each set of 5 stained slides per sample per staining day was randomized and evaluated by a total of 6 readers (2 readers/site) for a HER2-low status. The HER2-low status results for all readers, sites and days for the samples were combined and analyzed versus the reader modes for the same samples to determine the overall reproducibility of HER2-low

status. The summary of the agreement rates across all evaluable observations, using the sample-level reader modes for HER2-low status as the reference can be found inTable 16.

Table 16. Inter-laboratory reproducibility for overall agreement rates for VENTANA HER2 (4B5) Assay in breast carcinoma with HER2-low scoring.

Inter-Laboratory Reproducibility	Agreement			
	Type	n/N	%	95% CI
Primary Analysis/Overall	PPA	407/420	96.9	(93.6, 99.3)
	NPA	405/420	96.4	(92.2, 100.0)
	OPA	812/840	96.7	(94.0, 98.9)
Site- Stratified	PPA	407/420	96.9	(93.6, 99.3)
	NPA	405/420	96.4	(92.2, 100.0)
	OPA	812/840	96.7	(94.0, 98.9)
Reader-Stratified	PPA	412/425	96.9	(94.8, 98.7)
	NPA	405/415	97.6	(94.9, 100.0)
	OPA	817/840	97.3	(95.2, 98.9)

Note: Two-sided 95% CI calculated using the percentile bootstrap method with 2000 replicates stratified by case qualification score bin.

Note: For the purposes of study analysis, HER2 scores 0 and 3+ were grouped together as negative cases because they were ineligible for the clinical trial investigating HER2-low breast cancer. HER2 scores of 1+ and 2+ were grouped together as positive cases as they were eligible or potentially eligible for the clinical trial.

In addition, pairwise comparisons of HER2 (4B5) status were made between-sites, between-readers, and between-days. As summarized in Table 17, the assay was reproducible across 5 days, 3 sites, and 6 readers.

Table 17. Inter-laboratory reproducibility pairwise agreement rates for VENTANA HER2 (4B5) Assay in breast carcinoma with HER2-low scoring.

Inter-Laboratory Reproducibility	Agreement			
	Type	n/N	%	95% CI
Between-Site	APA	7982/8440	94.6	(90.3, 97.9)
	ANA	7902/8360	94.5	(90.6, 98.0)
	OPA	7942/8400	94.5	(90.5, 98.0)
Between-Reader	APA	402/422	95.3	(91.7, 98.1)
	ANA	398/418	95.2	(91.8, 98.1)
	OPA	400/420	95.2	(91.9, 98.1)
Between-Day	APA	1608/1688	95.3	(91.5, 98.2)
	ANA	1592/1672	95.2	(91.8, 98.2)
	OPA	1600/1680	95.2	(91.8, 98.2)

Note: Two-sided 95% CI calculated using the percentile bootstrap method with 2000 replicates stratified by case qualification score bin

Note: For the purposes of study analysis, HER2 scores 0 and 3+ were grouped together as negative cases because they were ineligible for the clinical trial investigating HER2-low breast cancer. HER2 scores of 1+ and 2+ were grouped together as positive cases as they were eligible or potentially eligible for the clinical trial.

Analytical Performance in HER2-positive Breast Cases

Concordance Between BenchMark ULTRA and BenchMark ULTRA PLUS Instruments for HER2-positive

Three laboratories participated in a concordance study to evaluate performance equivalence between the BenchMark ULTRA instrument and the BenchMark ULTRA PLUS instrument. For HER2-positive analysis, HER2 IHC score of 2+ or 3+ is defined as HER2-positive and a score of 0 or 1+ is defined as HER2 Negative. For HER2-positive statistical analysis, 160 (80 positive and 80 negative, including 16 borderline cases) of the cases were pre-selected for analysis prior to pathologist reads. Tissue slides from all cases were stained with a negative reagent control and VENTANA HER2 (4B5) Assay at an internal Roche laboratory on a BenchMark ULTRA instrument using the recommended staining protocol. Unstained tissue slides from all cases were randomized and equally distributed for staining on a BenchMark ULTRA PLUS instrument using the recommended VENTANA HER2 (4B5) staining protocol. Blinded to case status, one reader per site read the BenchMark ULTRA PLUS stained slides from their site and determined the HER2 (4B5) status. The results were analyzed by Roche. The results are summarized in Table 18.

Table 18. Pooled Agreement of HER2-positive status for Cases Stained with VENTANA HER2 (4B5) Assay on the BenchMark ULTRA versus BenchMark ULTRA PLUS Instrument.

BenchMark ULTRA PLUS	BenchMark ULTRA				
	Roche Reader= Positive, External Reader= Positive	Roche Reader= Positive, External Reader= Negative	Roche Reader= Negative, External Reader= Positive	Roche Reader= Negative, External Reader= Negative	Total
Positive	245	18	11	13	287
Negative	6	13	5	327	351
Total	251	31	16	340	638
Percent Positive % (n/N)	97.6 (245/251)	58.1 (18/31)	68.8 (11/16)	3.8 (13/340)	N/A
	n/N			% (95% CI)	
PPA	(263/282)			93.3 (89.3, 96.6)	
NPA	(332/356)			93.3 (89.7, 96.5)	
OPA	(595/638)			93.3 (90.8, 95.6)	

Note: Two-sided 95% CI calculated using the percentile bootstrap method with 2000 replicates stratified by IHC qualification score bin (0, 1+, 2+, 3+).

Note: The pooled agreement included all cases and ULTRA PLUS readers.

Note: PPA = Positive Percent Agreement; NPA = Negative Percent Agreement; OPA = Overall Percent Agreement.

Inter-Laboratory Reproducibility Study- BenchMark ULTRA PLUS for HER2-positive

An Inter-Laboratory Reproducibility Study of the VENTANA HER2 (4B5) Assay was completed to demonstrate reproducibility of the assay to determine HER2-positive status of breast carcinoma cases. The study included 28 de-identified, archival, FFPE breast carcinoma tissue specimens run across three BenchMark ULTRA PLUS instruments on each of five non-consecutive days over 20 days at three external laboratories. The specimens represented the range of staining of the VENTANA HER2 (4B5) Assay.

Each set of 5 stained slides per sample per staining day was randomized and evaluated by a total of 6 readers (2 readers/site) for a HER2-positive status. The HER2-positive status results for all readers, sites and days for the samples were combined and analyzed versus the reader modes for the same samples to determine the overall reproducibility of HER2-positive status. The summary of the agreement rates across all evaluable observations, using the sample-level reader modes for HER2-positive status as the reference can be found in Table 19.

Table 19. Inter-laboratory reproducibility for overall agreement rates for VENTANA HER2 (4B5) Assay in breast carcinoma with HER2-positive scoring.

Inter-Laboratory Reproducibility	Agreement			
	Type	n/N	%	95% CI
Primary Analysis/Overall	PPA	411/420	97.9	(95.7, 99.5)
	NPA	410/420	97.6	(94.3, 100.0)
	OPA	821/840	97.7	(96.0, 99.3)
Site- Stratified	PPA	411/420	97.9	(95.7, 99.5)
	NPA	410/420	97.6	(94.3, 100.0)
	OPA	821/840	97.7	(96.0, 99.3)
Reader-Stratified	PPA	413/420	98.3	(96.9, 99.5)
	NPA	412/420	98.1	(95.8, 100.0)
	OPA	825/840	98.2	(96.9, 99.4)

Note: Two-sided 95% CI calculated using the percentile bootstrap method with 2000 replicates stratified by case qualification score bin

Note: For the purposes of study analysis, HER2 IHC scores of 0 and 1+ were grouped together as negative and HER2 IHC scores of 2+ and 3+ were grouped together as positive.

In addition, pairwise comparisons of HER2 (4B5) status were made between-sites, between-readers, and between-days. As summarized in Table 20, the assay was reproducible across 5 days, 3 sites, and 6 readers.

Table 20. Inter-laboratory reproducibility pairwise agreement rates for VENTANA HER2 (4B5) Assay in breast carcinoma with HER2-positive staining.

Inter-Laboratory Reproducibility	Agreement			
	Type	n/N	%	95% CI
Between-Site	APA	8074/8420	95.9	(92.8, 98.6)
	ANA	8034/8380	95.9	(92.5, 98.7)
	OPA	8054/8400	95.9	(92.7, 98.6)
Between-Reader	APA	402/421	95.5	(92.0, 98.6)
	ANA	400/419	95.5	(91.6, 98.6)
	OPA	401/420	95.5	(91.9, 98.6)
Between-Day	APA	1634/1684	97.0	(95.0, 98.9)
	ANA	1626/1676	97.0	(94.8, 98.9)
	OPA	1630/1680	97.0	(95.0, 98.9)

Note: Two-sided 95% CI calculated using the percentile bootstrap method with 2000 replicates stratified by case qualification score bin

Note: For the purposes of study analysis, HER2 IHC scores of 0 and 1+ were grouped together as negative and HER2 IHC scores of 2+ and 3+ were grouped together as positive.

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 21. Site A vs. Site B Inter-laboratory Agreement Rates 3x3 Analysis–clone 4B5 BenchMark ULTRA instrument with *ultra*View Universal DAB Detection Kit.

Site A	Site B			
	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 (%) (95% CI)				43/48 (**89.6**) (77.8-95.5)

Table 22. 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 (%)(95% CI)				42/48 (**87.5**) (75.3-94.1)

Table 23. 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 (%) (95% CI)				44/48 (**91.7**) (80.4-96.7)

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 24. 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 25. 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 26. 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 iVIEW 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 27. 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 28. 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 29. 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.

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

*ultra*View 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	% (95% CI)	
Positive percent agreement	21/21	100 (84.5-100)	
Negative percent agreement	189/189	100 (98.0-100)	
Overall percent agreement	210/210	100 (98.2-100)	

Table 31. 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 32. Overall clinical assessment agreement between sites: gastric carcinoma, all evaluable cases.

BenchMark ULTRA instrument	Percent Overall Agreement (positive and negative cases)	Percent Overall Agreement (including equivocal cases)
Site A vs Site B: n/N (%) (95% CI)	30/30 (100%) (88.6 – 100)	38/42 (90.5%) (77.9 – 96.2)
Site A vs Site C: n/N (%) (95% CI)	30/30 (100%) (88.6 – 100)	35/42 (83.3%) (69.4 – 91.7)
Site B vs Site C: n/N (%) (95% CI)	30/30 (100%) (88.6 – 100)	31/42 (73.8%) (58.9 – 84.7)
BenchMark XT instrument	**Percent Overall Agreement (positive and negative cases)**	**Percent Overall Agreement (including equivocal cases)**
Site A vs Site B: n/N (%) (95% CI)	31/31 (100%) (89.0 – 100.0)	36/43 (83.7%) (70.0 – 91.9)
Site A vs Site C: n/N (%) (95% CI)	31/31 (100%) (89.0 – 100.0)	36/43 (83.7%) (70.0 – 91.9)
Site B vs Site C: n/N (%) (95% CI)	31/31 (100%) (89.0 – 100.0)	35/43 (81.4%) (67.4 – 90.3)

Table 33. Overall clinical assessment agreement between platforms: gastric carcinoma all evaluable cases.

BenchMark ULTRA instrument vs BenchMark XT instrument	Percent Overall Agreement (positive and negative cases)	Percent Overall Agreement (including equivocal cases)
Site A: n/N (%) (95% CI)	40/40 (100%) (91.2 – 100)	42/44 (95.5%) (84.9 – 98.7)
Site B: n/N (%) (95% CI)	34/34 (100%) (89.8 – 100)	37/42 (88.1%) (75.0 – 94.8)
Site C: n/N (%) (95% CI)	32/32 (100%) (89.3 – 100)	38/44 (86.4%) (73.3 – 93.6)

Table 34. 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 iVIEW 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 iVIEW DAB Detection Kit 96.6% (92.7-98.4), BenchMark GX instrument versus BenchMark XT instrument with iVIEW 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 iVIEW DAB Detection Kit 80.0% (54.8-93.0), BenchMark GX instrument versus BenchMark XT instrument with iVIEW 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 iVIEW DAB Detection Kit 98.1% (94.6-99.4), BenchMark GX instrument versus BenchMark XT instrument with iVIEW 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 35. 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 36. BenchMark GX instrument vs. BenchMark XT instrument Inter-Platform Agreement Rates with ultraView 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 37. BenchMark instrument vs. BenchMark XT instrument Inter-Platform Agreement Rates with iVIEW 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 38. BenchMark GX instrument vs. BenchMark XT instrument Inter-Platform Agreement Rates with iVIEW 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)	

CLINICAL PERFORMANCE

HER2-low Breast Cancer

Clinical Outcome Study- DESTINY-BREAST04

DESTINY-BREAST04 was a phase III multicenter, randomized, open-label, active controlled trial evaluating the safety and efficacy of trastuzumab deruxtecan (ENHERTU®) in unresectable and/or metastatic breast cancer subjects that express low levels of HER2.

In order to be eligible for study inclusion, tumors were required to demonstrate low levels of HER2 expression determined using IHC with the anti-HER2 (4B5) antibody.

A tumor with a HER2 IHC score of 1+ was considered to indicate a HER2-low status. A tumor was also considered HER2-low if the HER2 IHC score was 2+ and reflex testing with the INFORM HER2 Dual ISH assay indicated the absence of HER2 gene amplification (ISH-). Enrolled patients were randomized in a 2:1 ratio to treatment with trastuzumab deruxtecan (ENHERTU®) or with the chemotherapy treatment of physician's choice. The centrally obtained HER2-low score (IHC 1+ or IHC 2+/ISH-) was one of 3 stratification factors used for patient randomization in that study.

Efficacy analyses were performed in the full analysis set and the hormone receptor positive population (positive for estrogen receptor and/or progesterone receptor).

In the primary analysis, progression-free survival (PFS) based on blinded independent central review (BICR) assessment was analyzed in the hormone receptor positive subset with stratification by centrally assessed HER2-low status/score (IHC 1+ or IHC 2+/ISH-), number of prior lines of chemotherapy (1 or 2), and prior cyclin-dependent (CDK)4/6 inhibitor treatment (yes or no). Trastuzumab deruxtecan (ENHERTU®) treatment was associated with a statistically significant and clinically meaningful increase in PFS as well as overall survival (OS) in this population compared with the physician's treatment of choice.

Table 39. PFS and OS per BIRC in the Hormone Receptor-positive Population and Full Analysis Set (DESTINY-BREAST04)

	Hormone Receptor-positive Population		Full Analysis Set	
Parameter	Trastuzumab deruxtecan (ENHERTU®) N = 331	Treatment of Physician Choice N = 163	Trastuzumab deruxtecan (ENHERTU®) N = 373	Treatment of Physician Choice N = 184
Median PFS a (95% CI)	10.1 (9.5, 11.5)	5.4 (4.4, 7.1)	9.9 (9.0, 11.3)	5.1 (4.2, 6.8)
Hazard Ratio b (95% CI)	0.51 (0.40, 0.64)		0.50 (0.40, 0.63)	
P-value c	< 0.0001		< 0.0001	
Overall Survival (OS)				
Median OS a (95% CI)	23.9 (20.8, 24.8)	17.5 (15.2, 22.4)	23.4 (20.0, 24.8)	16.8 (14.5, 20.0)
Hazard Ratio b (95% CI)	0.64 (0.48, 0.86)		0.64 (0.49, 0.84)	
P-value c	0.0028		0.0010	

CI = confidence interval, PFS = progression-free survival, OS = overall survival

[a] Median PFS and OS are estimates from Kaplan-Meier analysis. Two-sided 95 CIs for median PFS and OS were computed using the Brookmeyer-Crowley method..

[b] Based on stratified Cox proportional hazards model. Stratification factors were HER2-low score, number of prior lines of chemotherapy, and either prior cyclin-dependent kinase 4/6 inhibitor treatment (for full analysis set and hormone receptor-positive) or hormone receptor/ cyclin-dependent kinase status (for full analysis set).

[c] Two-sided P-value from stratified log-rank test.

HER2-positive Breast Cancer

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 diagnostic tests. Six investigators participated in the study. Two independent cohorts of invasive breast cancer samples were used in the study: one with 178 samples from the Cleveland Clinic Foundation (Cohort 1), and one with 144 samples collected by IMPATH Predictive Oncology from multiple international sites (Cohort 2). Two sets of three different investigators evaluated the 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.[42,43,44] 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. Clinically significant results (positive/negative) from the HER2 (4B5) IHC assay and the PATHWAY anti-HER2 (CB11) IHC assay in the two cohorts are shown below:

Table 40. Clinically Significant Scores: IHC Assays for Cohort 1

HER2 (4B5) antibody	PATHWAY anti-HER2 (CB11) antibody		
	Positive	Negative	Total
Positive	86	5	91
Negative	7	80	87
Total	93	85	178
	n/N	% (95% Confidence Interval)	
Positive Percent Agreement	86/93	92.5 (85.2-96.9)	
Negative Percent Agreement	80/85	94.1 (86.8-98.1)	
Overall Percent Agreement	166/178	93.3 (88.5-96.4)	
Clinically significant results were considered IHC positive (2+ and 3+) and negative (0+ and 1+)			

Table 41. Clinically Significant Scores: IHC Assays for Cohort 2

HER2 (4B5) antibody	PATHWAY anti-HER2 (CB11) antibody		
	Positive	Negative	Total
Positive	69	22	91
Negative	0	53	53
Total	69	75	144
	n/N	% (95% Confidence Interval)	
Positive Percent Agreement	69/69	100 (97.5-100)	
Negative Percent Agreement	53/75	70.6 (58.5-80.1)	
Overall Percent Agreement	122/144	84.7 (78.2-90.0)	
Clinically significant results were considered IHC positive (2+ and 3+) and negative (0+ and 1+)			

The IHC-based results from the HER2 (4B5) IHC assay were also compared to results from the PathVysion HER2 FISH assay. IHC positive results correspond to cases with an IHC score of 2+ or 3+ and FISH positive results correspond to cases that demonstrated amplification of the HER2 gene. Agreement data for the clone 4B5 IHC assay compared to FISH results in the two cohorts are shown below:

Table 42. Clinically Significant Agreement: IHC to FISH for Cohort 1

HER2 (4B5) antibody	n/N	% (95% Confidence Interval)
Positive Percent Agreement	83/93	89.2 (82.5-95.1)
Negative Percent Agreement	77/85	90.6 (84.0-96.4)
Overall Percent Agreement	160/178	90.0 (85.4-93.6)

Table 43. Clinically Significant Agreement: IHC to FISH for Cohort 2

HER2 (4B5) antibody	n/N	% (95% Confidence Interval)
Positive Percent Agreement	80/86	93.0 (87.9-96.3)
Negative Percent Agreement	47/58	81.0 (73.4-86.0)
Overall Percent Agreement	127/144	88.2 (82.1-92.2)

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

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

Concordance to enrollment assays for cohorts from PERJETA and KADCYLA studies was determined by staining of trial specimens with VENTANA HER2 (4B5) Assay. A total of 2753 specimens evaluated for the PERJETA trial and 99 specimens evaluated for the KADCYLA trial were stained with VENTANA HER2 (4B5) Assay. 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.

Study	Clone 4B5 Score [b]	Dako HER2 Status [a,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)

Clinical Outcome Study – KATHERINE

The performance of HER2 clone 4B5 and INFORM HER2 Dual ISH DNA Probe Cocktail (INFORM HER2 Dual ISH assay) were investigated in KATHERINE (BO27938), a randomized, multicenter, open-label Phase III study to evaluate the efficacy and safety of trastuzumab emtansine (KADCYLA) versus trastuzumab (Herceptin) as adjuvant therapy for patients with HER2-positive primary breast cancer who have residual tumor present pathologically in the breast or axillary lymph nodes following preoperative therapy (NCT01772472).

Patient samples were stained with clone 4B5 and/or INFORM HER2 Dual ISH and evaluated for staining acceptability and HER2 status. Overall, most specimens were pre-treatment biopsy (80.9%), collected primarily as a biopsy (75.3%) or via surgical methods (24.3%). More specimens displayed ductal neoplastic subtype (95.4%), and most were not obtained from a metastatic sample (96.2%).

KATHERINE enrolled 1486 patients with HER2-positive, early breast cancer with residual invasive tumor in the breast and/or axillary lymph nodes following taxane and trastuzumab-based therapy as part of a neoadjuvant regimen before trial enrollment. Patients received radiotherapy and/or hormonal therapy concurrent with study treatment as per local guidelines. Breast tumor samples were required to show HER2 overexpression defined as 3+ IHC or ISH amplification ratio ≥ 2.0 determined at a central laboratory. Patients were randomized (1:1) to receive trastuzumab or KADCYLA. Randomization was stratified by clinical stage at presentation, hormone receptor status, preoperative HER2-directed therapy (trastuzumab, trastuzumab plus additional HER2-directed agent[s]), and pathological nodal status evaluated after preoperative therapy.

The primary efficacy endpoint of the KATHERINE study was invasive disease free survival (IDFS). IDFS was defined as the time from the date of randomization to first occurrence of ipsilateral invasive breast tumor recurrence, ipsilateral local or regional invasive breast cancer recurrence, distant recurrence, contralateral invasive breast cancer, or death from any cause.

A clinically meaningful and statistically significant improvement in IDFS was observed in patients whose breast cancer samples were identified as HER2-positive with the clone 4B5 IHC assay, who received trastuzumab emtansine (KADCYLA) compared with trastuzumab (Herceptin) (HR = 0.43, 95% CI [0.32, 0.58]), corresponding to a 57% reduction in risk of an IDFS event. Efficacy results for the IHC positive subgroup are presented in Table 48.

Data analysis also shows that with or without the adjustment for differential sampling in the study population due to local test prescreening, the drug efficacy estimates are similar.

Table 48. Efficacy results from KATHERINE for the IHC Positive Subgroup.

	KADCYLA N = 573	Trastuzumab N = 559
Primary Endpoint	Invasive Disease Free Survival (IDFS) [a]	
Number (%) of patients with event	64 (11.2%)	130 (23.3%)
HR [95% CI]	0.43 [0.32, 0.58]	
3-year event-free rate % [b]	89.0	75.7

[a] Data from first interim analysis

[b] 3-year event-free rate derived from Kaplan-Meier estimates

Data from the KATHERINE study show that adjuvant trastuzumab emtansine (KADCYLA) demonstrated a clear treatment benefit compared with adjuvant trastuzumab (Herceptin) in patients with HER2-positive early breast cancer with residual disease after completion of neoadjuvant treatment. The HER2 clone 4B5 and INFORM HER2 Dual ISH assays are useful in identifying those patients likely to benefit from trastuzumab emtansine (KADCYLA) treatment.

Gastric Cancer

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 stained by both assays and (after removing duplicate cases) 398 unique cases 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+. Agreement rates between clone 4B5 and HercepTest, for both cohorts studied are provided in the table below.

Table 49. 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

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 tissue sections wash off the slide, slides should be checked to ensure that they are positively charged.
5. 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.
6. 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 elabdoc.roche.com/symbols for more information).

GTIN	Global Trade Item Number
Rx only	For USA: Caution: Federal law restricts this device to sale by or on the order of a physician.

REVISION HISTORY

Rev	Updates
C	Updates to Staining Procedure section and Scoring conventions for gastric carcinoma on Table 7. Corrections to terminology and punctuation. Added BenchMark ULTRA PLUS instrument.

INTELLECTUAL PROPERTY

For USA: Rx only

CONTACT INFORMATION

Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany
+800 5505 6606

www.roche.com

CE 0123