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

REF 790-2991

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IVD Σ 50

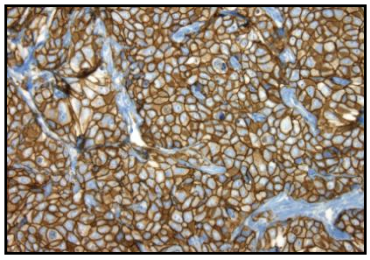

Figure 1. PATHWAY anti-HER2 (4B5) antibody staining in breast carcinoma.

INTENDED USE

PATHWAY anti-HER-2/neu (4B5) Rabbit Monoclonal Primary Antibody (PATHWAY anti-HER2 (4B5) antibody) is a rabbit monoclonal antibody intended for laboratory use for the semi-quantitative detection of HER2 antigen by immunohistochemistry (IHC) in sections of formalin-fixed, paraffin-embedded normal and neoplastic breast tissue using the *ultra*View Universal DAB Detection Kit on a BenchMark ULTRA instrument.

This IHC device is indicated for identifying breast cancer patients who are eligible for treatment with Herceptin® (IHC 3+ or IHC 2+/ISH amplified), KADCYLA® (IHC 3+ or IHC 2+/ISH amplified) or ENHERTU® (IHC 1+ or IHC 2+/ISH non-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

PATHWAY anti-HER2 (4B5) antibody is a rabbit monoclonal antibody produced against the internal domain of the c-erbB-2 oncoprotein (HER2). The c-erbB-2 oncoprotein was cloned and characterized by Akiyama, et al in 1986.[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 by Akiyama et al for 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 immunohistochemical staining of formalin-fixed, paraffin-embedded 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 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]

CLINICAL SIGNIFICANCE

Breast cancer is the most commonly diagnosed cancer in women worldwide.[12] Early detection and appropriate treatment selection can significantly affect overall survival.[7,13]

Approximately 15 - 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.[14,15] HER2-positive status has defined a subgroup of breast cancer patients who benefit from HER2-targeted therapy for more than 20 years.[7,13] The HER2-positive population has historically been defined as those patients that demonstrate HER2 protein overexpression assessed by IHC based on a semi-quantitative IHC scoring system (0, 1+, 2+ and 3+) and/or gene amplification assessed by in-situ hybridization (ISH).[13] 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.[13]

On-market therapeutic drugs, including trastuzumab (Herceptin®) and trastuzumab emtansine (KADCYLA®), have demonstrated clinical benefit in HER2-positive breast cancer patients by arresting, and in some cases reversing the growth of their cancer.[16,17,18] Trastuzumab is a humanized monoclonal antibody that binds to HER2 protein on the cell surface and disrupts HER2-mediated signal transduction.[16] Trastuzumab emtansine is an antibody-drug conjugate composed of trastuzumab and the cytotoxic agent DM1 conjugated through a non-cleavable linker.[18] Only patients with HER2-positive breast cancer should benefit from treatment with trastuzumab (Herceptin®) 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+ without confirmatory gene amplification) are typically considered HER2-negative and excluded from HER2-targeted treatment options.[8] Recently, benefit has been observed with the anti-HER2 treatment fam-trastuzumab deruxtecan-nxki (ENHERTU®) in breast cancer patients with low levels of HER2 expression.[19,20,21] Fam-trastuzumab deruxtecan-nxki 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 determination of HER2 status in breast cancer patients are important to aid the clinician in determination of therapy with trastuzumab (Herceptin®), trastuzumab emtansine (KADCYLA®) or fam-trastuzumab deruxtecan-nxki (ENHERTU®).[11] The immunohistochemical detection of HER2 protein expression may be used as an aid in the assessment of breast cancer patients for whom the treatments Herceptin®, KADCYLA® or ENHERTU® are being considered.

PRINCIPLE OF THE PROCEDURE

PATHWAY anti-HER2 (4B5) antibody is a rabbit monoclonal antibody, which binds to HER2 in formalin-fixed, paraffin-embedded (FFPE) tissue sections. The specific antibody is located by a cocktail of enzyme-labeled secondary antibodies that recognize rabbit immunoglobulins followed by the addition of 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 BenchMark ULTRA instrument washes the sections to stop the reaction and to remove unbound material that would hinder the desired reaction in subsequent steps. It also applies Liquid Coverslip, which minimizes evaporation of the aqueous reagents from the specimen slide.

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

The use of pre-diluted PATHWAY anti-HER2 (4B5) antibody and ready-to-use *ultra*View Universal DAB Detection Kit, in combination with a BenchMark ULTRA instrument,

reduces the possibility of human error and inherent variability resulting from individual reagent dilution, manual pipetting, and manual reagent application.

REAGENT PROVIDED

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

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

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

Specific antibody concentration is approximately 6 µg/mL. There is no known irrelevant antibody reactivity observed in this product.

PATHWAY anti-HER2 (4B5) antibody is a rabbit IgG diluted from tissue culture supernatants.

Refer to the *ultra*View Universal DAB Detection Kit package insert 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 General 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 package insert 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, Superfrost Plus [VWR Cat. No. 48311-703 or equivalent]
3. CONFIRM Negative Control Rabbit Ig (Cat. No. 760-1029) (negative reagent control)
4. *ultra*View Universal 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. ULTRA LCS (Predilute) (Cat. No. 650-210 / 05424534001) for BenchMARK ULTRA instrument
8. ULTRA Cell Conditioning Solution (ULTRA CC1) (Cat. No. 950-224 / 05424569001)
9. Hematoxylin II (Cat. No. 790-2208 / 05277965001)
10. Bluing Reagent (Cat. No. 760-2037 / 05266769001)
11. Permanent Mounting Medium
12. Cover glass
13. Automated coverslipper
14. General purpose laboratory equipment
15. BenchMark ULTRA Instruments

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 ULTRA instruments. Slides should be stained immediately, as antigenicity of cut tissue sections may diminish over time. The recommended tissue fixative is 10% neutral buffered formalin.[22] The amount used is 15 to 20 times the volume of tissue. No fixative will penetrate more than 2 to 3 mm of solid tissue or 5 mm of porous tissue in a 24-hour period. A 3 mm or smaller section of tissue should be fixed no less than 4 hours and no more than 8 hours. Fixation can be performed at room temperature (15-25°C).[23]

Properly fixed and embedded tissues (tissue blocks) expressing the antigen will remain stable for at least 2 years if stored in a cool location (15-25°C). The Clinical Laboratory Improvement Act (CLIA) of 1988, 42CFR493.1259(b) requires that "The laboratory must retain stained slides at least 10 years from the date of examination and retain specimen blocks at least 2 years from the date of examination."

Approximately 4-5 µm thick sections should be cut and picked up on glass slides. The slides should be Superfrost Plus or equivalent. Tissue should be air dried by placing the slides at ambient temperature overnight.[23] Studies at Ventana indicate that air dried cut tissue and cell line sections stored at 2-8°C are stable for a minimum of 45 days. However, each laboratory should validate the cut slide stability for their own procedures and environmental storage conditions.

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

WARNINGS AND PRECAUTIONS

1. For *in vitro* diagnostic (IVD) use.
2. For professional use only.
3. **CAUTION**: In the United States, Federal law restricts this device to sale by or on the order of a physician. (Rx Only)
4. Do not use beyond the specified number of tests.
5. Positively charged slides may be susceptible to environmental stresses resulting in inappropriate staining. Ask your Roche representative for more information on how to use these types of slides.
6. 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.
7. Avoid contact of reagents with eyes and mucous membranes. If reagents come in contact with sensitive areas, wash with copious amounts of water.
8. Avoid microbial contamination of reagents as it may cause incorrect results.
9. When used according to instructions, this product is not classified as a hazardous substance. The preservative in the reagent is sodium azide. Symptoms of overexposure to sodium azide include skin and eye irritation, and irritation of mucous membranes and upper respiratory tract. The concentration of sodium azide in this product is 0.05% and does not meet the OSHA criteria for a hazardous substance. 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.[24] Systemic allergic reactions are possible in sensitive individuals.
10. For further information on the use of this device, refer to the BenchMark ULTRA instrument User Guide, and instructions for use of all necessary components located at dialog.roche.com
11. Consult local and/or state authorities with regard to recommended method of disposal.
12. Product safety reagent labels primarily follow EU GHS guidance. Safety Data Sheet available for professional user on request.
13. 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 PROCEDURES

VENTANA primary antibodies have been developed for use on BenchMark ULTRA instruments in combination with VENTANA detection kits and accessories. Refer to the table below for the staining protocol. PATHWAY anti-HER2 (4B5) antibody is approved for use in the United States when using the PATHWAY staining procedure and staining protocol. This antibody has been optimized for specific incubation times but the user must verify results obtained with this reagent. PATHWAY anti-HER2 (4B5) antibody should be allowed to stand at least 30 minutes at room temperature prior to use. The parameters for the automated procedures can be displayed, printed and edited according to the procedure in the instrument User Guide. Other operating parameters for the instrument have been preset at the factory. Refer to the appropriate VENTANA detection kit package insert for more details regarding IHC staining procedures.

Staining Procedure for HER2 Assessment

The staining protocol and procedure listed in Table 1 is appropriate for use in all HER2 screening of breast carcinoma cases. Deviating from the recommended staining protocol may produce invalid results, 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.

Table 1. Staining Protocol for PATHWAY anti-HER2 (4B5) antibody for HER2 assessment on a BenchMark ULTRA instrument

Procedure Type	PATHWAY anti-HER2 (4B5)
Protocol step	**Parameter input**
Staining Procedure:	U PATHWAY HER2 4B5
Deparaffinization	Selected, 4 minutes, 72°C
Cell Conditioning*	ULTRA CC1, 36 minutes, Mild (95°C)
***ultra*View DAB Detection Kit**	*ultra*View Inhibitor: 4 minutes, 36°C *ultra*View HRP Multimer: 8 minutes, 36°C *ultra*View DAB: 8 minutes, 36°C *ultra*View DAB H_2O_2: 8 minutes, 36°C *ultra*View Copper: 4 minutes, 36°C
Antibody (Primary)*	PATHWAY HER2 4B5 Ab- 12 Min, 36°C Or Neg Ctl Rbt Ig- 12 Min, 36°C
Counterstain	Hematoxylin II, 4 minutes, 36°C
Post Counterstain	Bluing, 4 minutes, 36°C

* Cell Conditioning (mild) and Antibody (12 min.) conditions are pre-programmed with this staining procedure and do not show up as a selectable step to the user.

QUALITY CONTROL PROCEDURES

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 biopsy, or surgical specimen, prepared or fixed as soon as possible in a manner identical to test sections.

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.

Examples of positive control tissues for this antibody are weakly positive breast carcinoma tissues.

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 (catalog # 781-2991). PATHWAY HER-2 4 in 1 Control Slides may be useful for a preliminary validation of the instrument used for staining slides with PATHWAY anti-HER2 (4B5) antibody. These four cell line controls are characterized by in situ hybridization for gene copy number, Table 2. When processed and stained appropriately, the cell lines should stain as described in the PATHWAY Her-2 4 in 1 Control Slide method sheet (package insert). If the indicated staining is not evident in the appropriate cores, especially the 1+ and 2+ controls, the staining of the tissues should be repeated.

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

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

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

Positive Tissue Control

A positive control tissue fixed and processed in the same manner as the patient specimens must be run for each set of test conditions and with every PATHWAY anti-HER2 (4B5) antibody staining procedure performed. This tissue could contain both positive staining cell/tissue components and negative cell/tissue components and serve as both the positive and negative control tissue. Control tissue should be fresh autopsy/biopsy/surgical specimens prepared and fixed as soon as possible in a manner identical to test sections. Such tissue may monitor all steps of the analysis, from tissue preparation through staining. Use of a tissue section fixed or processed differently from the test specimen provides control for all reagents and method steps except fixation and tissue preparation. A tissue with weak positive staining is more suitable than strong positive staining for optimal quality control and to detect minor levels of reagent degradation. Ideally a tissue which is known to have weak but positive staining should be chosen to ensure that the system is sensitive to small amounts of reagent degradation or problems with the IHC methodology. Generally, however, neoplastic tissue that is positive for HER2 is strongly positive due to the nature of the pathology (overexpression). An example of a positive control for PATHWAY anti-HER2 (4B5) antibody is a known weak HER2 positive invasive breast carcinoma (for example ductal or lobular). The positive staining tissue components (membrane of neoplastic cells) are used to confirm that the antibody was applied and the instrument functioned properly.

A known weak HER2 positive invasive breast carcinoma tissue may contain both positive and negative staining cells or tissue components and may serve as both the positive and negative control tissue.

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

Negative Tissue Control

The same tissue used for the positive tissue control (ductal or lobular invasive breast carcinoma) may be used as the negative tissue control. The non-staining components (surrounding stroma, lymphoid cells and blood vessels) should demonstrate absence of specific staining and provide an indication of specific background staining with the primary antibody. Use a tissue known to be fixed, processed and embedded in a manner identical to the patient sample(s) with each staining run to verify the specificity of PATHWAY anti-HER2 (4B5) antibody for demonstration of HER2, and to provide an indication of specific background staining (false positive staining).

Negative Reagent Control

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

Unexplained Discrepancies

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

Assay Verification

Prior to initial use of an antibody or staining system in a diagnostic procedure, the specificity of the antibody should be verified by testing it on a series of tissues with known immunohistochemistry performance characteristics representing known positive and negative tissues (refer to the Quality Control Procedures previously outlined in this section of the product insert and to the Quality Control recommendations of the College of American Pathologists Laboratory Accreditation Program, Anatomic Pathology Checklist,[26] or the CLSI Approved Guideline[27] or both documents). These quality control procedures should be repeated for each new antibody lot, or whenever there is a change in assay parameters. Breast cancer tissues with known HER2 status are suitable for assay verification.

STAINING INTERPRETATION / EXPECTED RESULTS

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

Positive Controls

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

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

Negative Tissue Controls

The negative tissue control should be examined after the positive tissue control to verify the specific labeling of the target antigen by the primary antibody. The absence of specific staining in the negative tissue control confirms the lack of antibody cross reactivity to cells or cellular components. If the tissue is counterstained, there may be staining around the outside of the cell, i.e., the interstitial spaces. If specific staining occurs in the negative tissue control, results with the patient specimen should be considered invalid.

Negative Reagent Controls

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

Patient Tissue

Patient specimens should be examined last. Positive staining intensity should be assessed within the context of any background staining of the negative reagent control. As with any immunohistochemical test, a negative result means that the antigen in question was not detected, not that the antigen is absent in the cells or tissue assayed. The morphology of each tissue sample should also be examined utilizing a hematoxylin and eosin stained section when interpreting any immunohistochemical result. The patient's morphologic findings and pertinent clinical data must be interpreted by a qualified pathologist.

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

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

Below is a quick reference chart for staining criteria. Refer to Interpretation Guide for PATHWAY anti-HER-2/*neu* (4B5) Rabbit Monoclonal Primary Antibody for a more detailed description with photographs of staining with PATHWAY anti-HER2 (4B5) antibody.

Table 3. Scoring Criteria for Intensity and Pattern of Cell Membrane Staining with PATHWAY anti-HER2 (4B5) Antibody

Staining pattern	HER2 (4B5) Score (Report to treating physician)	Recommended Reporting Status	Therapy
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 (fam-trastuzumab deruxtecan-nxki)
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), KADCYLA (trastuzumab emtansine)
Intense complete staining of the membrane in greater than 10% of the cancer cells	3+	HER2 positive / overexpression	

* Recommend re-reading by a second pathologist for cases with "faint, partial staining of the membrane" and %TC near the threshold of 10%, when the range of %TC is between 5%-25%

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

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. Any deviation from recommended test procedures may invalidate expected results. 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.[28] 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.[29]
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.[30]
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 on BenchMark ULTRA instruments and detection chemistries. Deviating from the recommended staining protocol in Table 1 may produce unacceptable Negative Reagent Control (NRC) samples and PATHWAY anti-HER2 (4B5) antibody-stained samples with a changed HER2 Score. Increased antibody incubation time is likely to produce unacceptable staining in the NRC, which would prevent the PATHWAY anti-HER2 (4B5) antibody sample from being evaluated. Decreased and increased cell conditioning times are likely to produce PATHWAY anti-HER2 (4B5) antibody samples with changed HER2 scores which may cause inappropriate treatment decisions for patients. Because of variation in tissue fixation and processing, it may be necessary to increase or decrease the primary antibody incubation time on individual specimens. For further information on fixation variables, refer to "Immunohistochemistry Principles and Advances".[31]
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. 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. All assays might not be registered on every instrument. Please contact your local Roche representative for more information.
5. 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. [31]

PERFORMANCE CHARACTERISTICS

ANALYTICAL PERFORMANCE

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

Sensitivity and Specificity

Analytical sensitivity was evaluated by characterizing HER2 prevalence (percent) among breast cancer tissue specimens in clinical trial DESTINY-Breast04.

Table 4. Prevalence* of HER2 IHC Scores in Clinical Trial DESTINY-Breast04

HER2 IHC Bin	n/N	%
0	267/1,303	20.5
1+	554/1,303	42.5
2+	440/1,303	33.8
3+	13/1.303	1.0
Not evaluable	29/1,303	2.2

*In different populations, prevalence of HER2 IHC scores can be different from the prevalence presented in Table 4

Analytical specificity was determined by staining multiple cases of normal and neoplastic human tissues with PATHWAY anti-HER2 (4B5) antibody. Staining results are listed in Table 5. and Table 6. The study showed no specific membrane staining for most normal tissues.

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

Any improper tissue handling during fixation, sectioning, embedding or storage which alters antigenicity weakens HER2 protein detection by PATHWAY anti-HER2 (4B5) antibody and may generate false negative results.

Specificity

Table 5. Specificity of PATHWAY anti-HER2 (4B5) antibody was determined by testing formalin-fixed, paraffin-embedded normal tissues.

Tissue	# positive / total cases	Tissue	# positive / total cases
Adrenal Gland	0/6	Ovary	0/6
Bladder	3/3*	Pancreas	0/6
Breast	0/14	Parathyroid	4/6**
Bone Marrow	0/3	Peripheral Nerve	2/6
Cardiac Pericardium	0/3	Prostate	1/6
Cerebrum	0/6	Rectum	0/6
Cerebellum	0/6	Salivary Gland	0/3
Cervix	0/5	Skeletal Muscle	0/6
Colon	0/46	Skin	0/6
Endocervix	0/1	Small Intestine	0/6
Endometrium	0/3	Spleen	0/6
Esophagus	1/6	Stomach	0/11
Heart	0/5	Testis	0/6
Hypophysis	0/5	Thymus Gland	0/5
Kidney	0/6	Thyroid	0/6
Liver	0/6	Tongue	0/3
Lung	0/6	Tonsil	3/6***
Lymph Node	0/12	Uterus	0/3
Mesothelium NOS	0/3		

* membranous staining of superficial umbrella cells

** focal membrane staining

*** focal staining of surface epithelial cells

NOS = Not otherwise specified

Sensitivity

Table 6. Sensitivity of PATHWAY anti-HER2 (4B5) antibody 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 Not Otherwise Specified (NOS) (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)	42/99
Carcinoma NOS (Breast)	1/4
B-cell Lymphoma NOS (Spleen)	0/1
Small cell carcinoma (Lung)	0/1
Squamous cell carcinoma (Lung)	0/1
Adenocarcinoma (Lung)	0/1
Carcinoma NOS (Lung)	0/2
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(GIST) (Small Intestine)	0/1
Adenocarcinoma (Colon)	0/32
Gastrointestinal Stromal Tumor (GIST) (Colon)	0/1
Carcinoma NOS (Colon)	1/3
Adenocarcinoma (Rectum)	1/5
Gastrointestinal Stromal Tumor (GIST) (Rectum)	0/1
Mesothelioma (Peritoneum)	0/1
B-Cell Lymphoma NOS (Lymph node)	0/2
Hodgkin lymphoma (Lymph node)	0/1
Lymphoma NOS	0/3
Urothelial carcinoma (Bladder)	1/1
Leiomyosarcoma (Bladder)	0/1
Osteosarcoma (Bone)	0/1
Pleomorphic rhabdomyosarcoma (Peritoneum)	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
Leiomyoma (Uterus)	0/1
Adenocarcinoma (Uterus)	0/1
Clear cell carcinoma (Uterus)	0/1
Squamous cell carcinoma (Cervix)	0/2
Embryonal rhabdomyosarcoma (Striated muscle)	0/1
Melanoma (Rectum)	0/1
Melanoma NOS	0/2
Basal cell carcinoma (Skin)	0/1
Squamous cell carcinoma (Skin)	1/1
Neurofibroma (Lumbar)	0/1
Neuroblastoma (Retroperitoneum)	0/1
Leiomyosarcoma (Smooth muscle)	0/1
Metastatic Adenocarcinoma (from Rectum)	0/1
Metastatic Adenocarcinoma (from Colon)	0/7
Metastatic mucinous adenocarcinoma (from Colon)	0/1
Carcinoid (NOS)	0/2
Leiomyoma NOS	0/2
Sarcoma NOS	0/2
Undifferentiated carcinoma NOS	0/1

Precision

For an evaluation of the precision of the PATHWAY anti-HER2 (4B5) antibody on BenchMark ULTRA, three precision studies were conducted: Intermediate Precision study, Reader (Pathologist) Precision study and Inter-Laboratory and Inter-Reader Precision (Reproducibility) study.

Intermediate Precision for HER2-low on BenchMark ULTRA

Twenty-four breast carcinoma cases spanning the HER2 IHC staining range were included in the intermediate precision study. The study design for evaluation of staining precision on breast carcinoma tissues stained with PATHWAY anti-HER2 (4B5) antibody included:.

- Three lots of PATHWAY anti-HER2 (4B5) antibody
- Three lots of *ultra*View DAB IHC Detection Kits
- Across three days
- Three BenchMark ULTRA instruments
- One pathologist, 2 replicates

All slides were blinded and randomized, and evaluated using the Criteria for Intensity and Pattern of Cell Membrane Staining with PATHWAY anti-HER2 (4B5) Antibody staining Each case had 18 results and a majority HER2 bin result was assigned based on 18 results. For each case, it was calculated a median %TC and range of %TC of 18 results. In addition, it was calculated percent Eligible with regard to HER2-low therapy. Among 24 cases, there were 3 cases with majority HER2 bin of 0, 10 cases with majority HER2 bin of 1+, 6 cases with majority HER2 bin of 2+ and 5 cases with majority HER2 bin of 3+. Results of this analysis were presented in the table below.

Table 7. Median and Range of %TC for Cases in the Intermediate Precision Study

Case	Majority HER2 Bin	Median %TC	Range %TC (Min-Max)	Percent Eligible
1	0	0.0	0 - 0	0 % (0/18)
2	0	0.0	0 - 0	0% (0/18)
3	0	1.0	1 - 2	0.0% (0/18)
4	1+	15.0	5 - 20	78% (14/18)
5	1+	15.0	10 - 20	94% (17/18)
6	1+	17.5	8 - 30	94% (17/18)
7	1+	20.0	15 - 20	100% (18/18)
8	1+	20.0	15 - 25	100% (18/18)
9	1+	20.0	15 - 35	100% (18/18)
10	1+	22.5	15 - 25	100% (18/18)
11	1+	25.0	15 - 35	100% (18/18)
12	1+	30.0	20 - 35	100% (18/18)
13	1+	50.0	35 - 50	100% (18/18)
14	2+	20.0	15 - 25	100% (18/18)
15	2+	20.0	15 - 35	100% (18/18)
16	2+	25.0	15 - 35	100% (18/18)
17	2+	35.0	15 - 50	100% (18/18)
18	2+	35.0	25 - 40	100% (18/18)
19	2+	50.0	50 - 50	100% (18/18)
20	3+	60.0	60 - 60	0% (0/18)
21	3+	75.0	75 - 80	0% (0/18)
22	3+	95.0	70 - 100	0% (0/18)
23	3+	100.0	95 - 100	0% (0/18)
24	3+	100.0	100 - 100	0% (0/18)

Twenty one (21) out of 24 cases had 18 results with the same type of staining ('No staining" or "Faint, partial staining" or "Weak to moderate complete staining" or "Intense complete staining"), variability of %TC values for 21 cases was evaluated and the following precision components were calculated: repeatability (within-pathologist), between-day, between-antibody kit, between-detection kit, between-instrument and total. Results are summarized in the table below:

Table 8. Precision Components for Cases in Intermediate Precision Study

Case	Majority HER2 Bin	Median %TC	SD					
			Repeatability (within-run)	Between-day	Between-antibody lot	Between-detection kit	Between-instrument	Total
1	0	0.0	0.00	0.00	0.00	0.00	0.00	0.00
2	0	0.0	0.00	0.00	0.00	0.00	0.00	0.00
3	0	1.0	0.00	0.00	0.00	0.58	0.58	0.82
4	1+	15.0	0.71	7.62	0.00	0.00	0.00	7.65
5	1+	15.0	1.67	0.00	0.00	2.64	2.20	3.82
6	1+	17.5	3.11	1.87	0.00	0.00	4.08	5.46
7	1+	20.0	1.18	1.18	0.00	2.04	0.00	2.64
8	1+	20.0	0.00	0.00	2.89	5.00	2.89	6.45
9	1+	20.0	N/A	N/A	N/A	N/A	N/A	N/A
10	1+	22.5	2.64	3.91	0.00	0.00	0.00	4.71
11	1+	25.0	3.33	0.83	6.77	3.54	2.89	8.86
12	1+	30.0	4.41	0.00	3.91	3.91	4.17	8.21
13	1+	50.0	3.54	5.77	0.00	0.00	0.00	6.77
14	2+	20.0	1.18	0.00	2.76	2.76	1.18	4.25
15	2+	20.0	N/A	N/A	N/A	N/A	N/A	N/A
16	2+	25.0	N/A	N/A	N/A	N/A	N/A	N/A
17	2+	35.0	5.77	9.13	0.00	8.29	0.00	13.62
18	2+	35.0	1.18	0.00	2.36	5.71	2.76	6.87
19	2+	50.0	0.00	0.00	0.00	0.00	0.00	0.00
20	3+	60.0	0.00	0.00	0.00	0.00	0.00	0.00
21	3+	75.0	0.00	0.00	2.89	0.00	2.89	4.08
22	3+	95.0	2.89	0.00	12.16	0.00	2.04	12.67
23	3+	100.0	1.18	0.00	2.76	0.00	2.36	3.82
24	3+	100.0	0.00	0.00	0.00	0.00	0.00	0.00

In addition, a qualitative analysis of different precision components was performed. 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. Results are summarized in Table 9.

Table 9. Repeatability and intermediate precision of PATHWAY anti-HER2 (4B5) antibody 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).

Reader Precision for HER2-low on BenchMark ULTRA

In the Reader Precision study, Between-Reader and Within-Reader components of precision were evaluated.. 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 PATHWAY anti-HER2 (4B5) Antibody staining (Table 3). The study included three readers (pathologist). Readers scored all specimens twice, with a minimum of two weeks between reads. Each case had 6 reads (2 reads by each of three readers). Data of the Reader precision study is presented in the table below.

Table 10. Results of the Reader Precision Study

Case Category	HER2 IHC	N of cases	N of reads	Results by HER2 IHC, %TC Category					Percent Results "Eligible"
				0, No staining	0, faint incomplete ≤10%	1+, faint incomplete >10%	2+, Weak to moderate complete	3+, intense complete	
No staining	0	6	36	100% (36/36)	0	0	0	0	0% (0/36)
No staining/ faint incomplete ≤10%	0	13	78	32% (25/78)	68% (53/78)	0	0	0	0% (0/78)
Faint incomplete ≤10%	0	7	42	0	100% (42/42)	0	0	0	0% (0/42)
Faint incomplete ≤10%/>10%	0/1+	7	42	0	79% (33/42)	21% (9/42)	0	0	21% (9/42)
Faint incomplete ≤10%/>10%	0/1+	9	54	0	28% (15/54)	72% (39/54)	0	0	72% (39/54)
Faint incomplete >10%	1+	5	30	0	0	100% (30/30)	0	0	100% (30/30)
Faint incomplete >10%/ weak to moderate complete	1+/2+	13	78	0	0	73% (57/78)	27% (21/78)	0	100% (78/78)
Faint incomplete >10%/ weak to moderate complete	1+/2+	11	66	0	0	38% (18/66)	62% (48/66)	0	100% (66/66)
Weak to moderate complete	2+	15	90	0	0	0	100% (90/90)	0	100% (90/90)
Weak to moderate complete/ Intense complete	2+/3+	3	18	0	0	0	67% (12/18)	33% (6/18)	67% (12/18)
Variable	0/1+/2+	2	12	0	25% (3/12)	50% (6/12)	25% (3/12)	0	75% (9/12)
Intense complete	3+	9	54	0	0	0	0	100% (54/54)	0% (0/54)

Fifty-two (52) out of 100 cases had 6 results with the same type of staining ("Faint, partial staining" or "Weak to moderate complete staining" or "Intense complete staining"), variability of %TC values for 52 cases was evaluated and following precision components were calculated: within-reader, between-reader and total. Results are summarized in the table below:

Table 11. Precision Components for Cases in Reader Precision Study

Case Category	HER2 IHC	N of cases	Range of median %TC	SD			Percent Results "Eligible"
				Within-Reader	Between-Reader	Total	
Faint incomplete ≤10%	0	7	3.0-6.5	1.8	1.2	2.2	0% (0/42)
Faint incomplete ≤10%/>10%	0/1+	7	2.5-7.5	3.5	3.4	4.9	21% (9/42)
Faint incomplete ≤10%/>10%	0/1+	9	8.0-25.0	18.5	3.4	18.8	72% (39/54)
Faint incomplete >10%	1+	5	11.5-37.5	17.9	13.5	22.5	100% 930/30)
Weak to moderate complete	2+	15	40.0-92.5	14.7	8.9	17.2	100% (90/90)
Intense complete	3+	9	37.5-99.5	13.8	8.5	16.2	0% (0/54)

In addition, a qualitative analysis of different precision components was performed. 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. The agreement for between-reader and within-reader precision components are summarized in 0.

Table 12. Within and Between-Reader Precision of the PATHWAY anti-HER2 (4B5) antibody 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).

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

An Inter-Laboratory Reproducibility Study of the PATHWAY anti-HER2 (4B5) antibody was conducted to evaluate reproducibility of the assay to determine HER2-low status of breast carcinoma cases. The study included 28 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 PATHWAY anti-HER2 (4B5) antibody.

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. Each case had 10 results per site (30 results in total). For each case, it was calculated a median %TC and range of %TC of 30 results. In addition, it was calculated percent Eligible with regard to HER2-low therapy. Results of this analysis for each case were presented in the table below.

Table 13. Results of the Inter-Laboratory Reproducibility Study

Case	Majority HER2 Bin	N of reads	0, No staining	0, faint incomplete ≤10%	1+, faint incomplete >10%	2+, weak to moderate complete	3+, intense complete	Percent Results "Eligible"			
								Site A	Site B	Site C	Overall
1	0	30	100% (30/30)	0	0	0	0	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
2	0	30	100% (30/30)	0	0	0	0	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
3	0	30	97% (29/30)	3% (1/30)	0	0	0	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
4	0	30	93% (28/30)	7% (2/30)	0	0	0	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
5	0	30	80% (24/30)	20% (6/30)	0	0	0	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
6	0	30	97% (29/30)	3% (1/30)	0	0	0	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
7	0	28	93% (26/28)	7% (2/28)	0	0	0	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
8	0	30	93% (28/30)	7% (2/30)	0	0	0	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
9	0	30	77% (23/30)	23% (7/30)	0	0	0	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
10	0	30	50% (15/30)	50% (15/30)	0	0	0	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
11	0	30	20% (6/30)	77% (23/30)	3% (1/30)	0	0	10% (1/10)	0% (0/10)	0% (0/10)	3% (1/30)
12	1+	30	0	10% (3/30)	90% (27/30)	0	0	70% (7/10)	100% (10/10)	100% (10/10)	90% (27/30)
13	1+	30	0	7% (2/30)	93% (28/30)	0	0	80% (8/10)	100% (10/10)	100% (10/10)	93% (28/30)
14	1+	30	0	0	87% (26/30)	13% (4/30)	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)

Case	Majority HER2 Bin	N of reads	0, No staining	0, faint incomplete ≤10%	1+, faint incomplete >10%	2+, weak to moderate complete	3+, intense complete	Percent Results "Eligible"			
								Site A	Site B	Site C	Overall
15	1+	30	0	3% (1/30)	97% (29/30)	0	0	100% (10/10)	100% (10/10)	90% (9/10)	97% (29/30)
16	1+	30	0	0	93% (28/30)	7% (2/30)	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)
17	1+	30	0	0	97% (29/30)	3% (1/30)	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)
18	1+	28	0	0	57% (16/28)	43% (12/28)	0	100% (8/8)	100% (10/10)	100% (10/10)	100% (28/28)
19	1+	30	0	3% (1/30)	80% (24/30)	17% (5/30)	0	90% (9/10)	100% (10/10)	100% (10/10)	97% (29/30)
20	2+	30	0	0	7% (2/30)	93% (28/30)	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)
21	2+	30	0	0	3% (1/30)	97% (29/30)	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)
22	2+	30	3% (1/30)	0	14% (4/30)	83% (25/30)	0	100% 10/10)	90% (9/10)	100% (10/10)	97% (30/30)
23	2+	30	0	0	0	100% (30/30)	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)
24	2+	30	0	0	13% (4/30)	87% (26/30)	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)
25	2+	28	0	0	7% (2/28)	89% (25/28)	4% (1/28)	100% (8/8)	90% (9/10)	100% (10/10)	96% (27/28)
26	3+	30	0	0	0	3% (1/30)	97% (29/30)	0% (0/10)	10% (1/10)	0% (0/10)	3% (1/30)
27	3+	30	0	0	0	0	100% (30/30)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
28	3+	30	0	0	0	0	100% (30/30)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)

Eight (8) out of 28 cases had 30 results with the same type of staining ("No staining" or "Faint, partial staining" or "Weak to moderate complete staining" or "Intense complete staining"), variability of %TC values for 8 cases was evaluated and following precision components were calculated: between-reader, between-day, between-site and total. Results are summarized in the table below:

Table 14. Precision Components for Cases in the Inter-Laboratory Reproducibility Study

Case	Case category	HER2 Bin	N of reads	Median %TC	Range %TC (Min-Max)	SD			
						Between-reader	Between-day	Between-site	Total
1	No staining	0	30	0.0	0-0	0.0	0.0	0.0	0.0
2	No staining	0	30	0.0	0-0	0.0	0.0	0.0	0.0
12	Faint incomplete ≤10%/>10%	0/1+	30	15.0	5-50	9.0	0.0	0.0	9.0
13	Faint incomplete ≤10%/>10%	0/1+	30	17.5	5-50	11.4	2.8	0.0	11.7
15	Faint incomplete ≤10%/>10%	0/1+	30	25.0	8-50	7.8	6.8	11.2	15.2
23	Weak to moderate complete	2+	30	60.0	20-90	12.1	6.2	19.4	23.7
27	Intense complete	3+	30	95.0	90-100	3.3	0.6	0.0	3.4
28	Intense complete	3+	30	95.0	90-100	2.6	0.0	0.0	2.6

In addition, a qualitative analysis of different precision components was performed. 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. Results of the analysis are presented in the table below.

Table 15. Inter-Laboratory Reproducibility for overall agreement rates for PATHWAY anti-HER2 (4B5) antibody 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).

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 16.

Table 16. Inter-Laboratory Reproducibility Pairwise Agreement Rates for the PATHWAY anti-HER2 (4B5) antibody 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)

Re-reading by Additional Pathologists for HER2-low Scoring

To decrease variability of HER2-low scoring for cases with "Faint incomplete staining" and %TC near the threshold of 10% [5% to 25%], re-reading of the slide by a second pathologist is recommended. The case result "Faint incomplete staining" and %TC between 5-25% by a pathologist should be adjudicated by one or two independent pathologists. The patient's final result with regard to "Eligibility" should be obtained by either a majority rule or by consensus among the pathologists.Repeatability and Intermediate Precision for HER2-positivity on BenchMark XT and BenchMark ULTRA.

Intra-run precision of staining on the BenchMark ULTRA and BenchMark XT instrument platforms was determined by staining three slides each of five breast cancer tissues with a score of 0, 1+, 2+, and 3+ HER-2 expression. For each case, three of 3 slides stained appropriately within a run and for all instrument platforms tested. Users should verify within run reproducibility results by staining several sets of serial sections with low, medium and high antigen density in a single run.

Inter-run and inter-platform precision of staining was determined by staining three slides each of five breast cancer tissues with scores of 0, 1+, 2+, and 3+ HER2 expression on three different instrument runs across the BenchMark and BenchMark XT instrument platforms. For each case, nine of 9 slides stained appropriately over three instrument runs and across all instrument platforms tested. Users should verify between run precision results by staining several sets of serial sections with low, medium and high antigen density on different days.

Lot-to-Lot Precision

Lot-to-Lot precision was determined by automated staining of 5 breast cancer tissues with scores of 0, 1+, 2+, and 3+ HER2 expression with 3 lots of PATHWAY anti-HER2 (4B5) antibody. Stained tissues were scored on a 0 to 3+ scale by three qualified readers. There was 100% agreement between lots and readers for the 3 slides and 5 tissues stained.

Inter-Laboratory and Inter-Reader Reproducibility for HER2-positivity on BenchMark XT Instrument

BenchMark XT Instrument Inter-laboratory staining and Inter-reader scoring reproducibility: Three laboratories, from separate institutions in the United States, participated in the inter-laboratory reproducibility study. Cut slides of 40 neutral buffered formalin-fixed invasive breast carcinoma cases [10 each from each HER-2 binning category (0-1+, 2+, 3+)] and six (6) PATHWAY HER-2 4 in 1 Control Slides were shipped to each of the sites for staining on a BenchMark XT instrument using the recommended staining protocol. Controls included the PATHWAY HER-2 4 in 1 Control Slides and a second slide of each case stained with negative Ig reagent. No sites experienced invalid runs, based upon the performance of the controls. The results were analyzed by Ventana. Thirty-four of forty (34/40) slides exhibited similar staining intensity across staining sites. Six samples (6/40 or 15%) varied by no more than 1 intensity level. Three (3/6) samples varied between 0 and 1+, which are both considered to be negative. Two samples (2/40 or 5%) varied between 2+ and 3+, and one sample (1/40) varied between 1+ and 2+. In all of the 40 cases (100%), a minimum of 2 of 3 pathologists agreed.

Performance Characteristics on BenchMark ULTRA Instrument Using *i*VIEW DAB Detection Kit or *ultra*View Universal DAB Detection Kit for HER2-positivity

BenchMark ULTRA Instrument Inter-laboratory Staining and Inter-day Reproducibility for HER2-positivity

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

The following Table 17, Table 26 and Table 27 are 3x3 presentations of results for each reader based on clinical score where 2+ and 3+ were separated:

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

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

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

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

Table 19. Site B vs. Site C Inter-laboratory Agreement Rates 3x3 Analysis – PATHWAY anti-HER2 (4B5) Antibody BenchMark ULTRA instrument with *ultra*View Universal DAB Detection Kit.

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

BenchMark ULTRA Instrument Inter day Staining Precision for HER2-positivity

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 PATHWAY anti-HER2 (4B5) antibody 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 to BenchMark XT Instrument for HER2-positivity

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 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 Controls Slides and a second slide of each case stained with negative Ig reagent. Stained cases from Site 1 and Site 2 were divided into four slide sets and provided, one set at a time, to three different qualified readers (pathologists), one reader at Site 1, one at Site 2, and one at Site 3. The pathologists, blinded to case status and staining platform, evaluated all four sets of slides and provided a clinical score (i.e. 0, 1+, 2+, 3+) for each case. The results were analyzed by Ventana. The PPA rates (and lower bound of the two-sided 95% confidence intervals) for PATHWAY anti-HER2 (4B5) antibody staining on the BenchMark ULTRA instrument versus the BenchMark XT instrument based on positive versus negative clinical assessment were 91.6% (85.9), 91.2% (85.3), and 94.9% (89.3) for Reader A, B, and C, respectively. The NPA rates (and lower bound of the two-sided 95% confidence intervals) for PATHWAY anti-HER2 (4B5) antibody staining on the Benchmark ULTRA instrument versus the BenchMark XT instrument based on positive versus negative clinical assessment were 91.9% (85.8), 93.8% (88.3), and 99.3% (96.3) for Reader A, B, and C, respectively. The OPA between the PATHWAY anti-HER2 (4B5) antibody using BenchMark ULTRA instrument versus BenchMark XT instrument based on 2x2 analysis of positive versus negative clinical assessment was 91.8%, 92.5%, and 97.4% per Reader A, B, and C, respectively. The 3x3 presentation of inter-platform agreement rates for each reader based on clinical score (0/1+, 2+, 3+) are shown in Table 20, Table 21, and Table 22.

Table 20. BenchMark ULTRA 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 21. BenchMark ULTRA 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 22. BenchMark ULTRA 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 Platform Comparison Study Specimens for HER2-positivity

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. The presentation of the pairwise agreement rates between readers for each platform are shown in Table 23 and Table 24.

Table 23. Benchmark ULTRA Instrument Inter-Pathologist Pairwise Agreement Rates

Comparison	Agreement Rate	% n/N
Reader A vs. B	PPA	94.7% (126/133)
	NPA	88.8% (119/134)
	OPA	91.8%
Reader A vs. C	PPA	98.2% (111/113)
	NPA	80.5% (124/154)
	OPA	88.8%
Reader B vs. C	PPA	98.2% (111/113)
	NPA	85.7% (132/154)
	OPA	91.0%
Reader B vs. A	PPA	89.4% (126/141)
	NPA	94.4% (119/126)
Reader C vs. A	PPA	78.7% (111/141)
	NPA	98.4% (124/126)
Reader C vs. B	PPA	83.5% (111/133)
	NPA	98.5% (132/134)

Table 24. Benchmark XT Instrument Inter-Pathologist Pairwise Agreement Rates

Comparison	Agreement Rate	% n/N
Reader A vs. B	PPA	94.9% (130/137)
	NPA	90.0% (117/130)
	OPA	92.5%
Reader A vs. C	PPA	98.3% (116/118)
	NPA	81.9% (122/149)
	OPA	89.1%
Reader B vs. C	PPA	98.3% (116/118)
	NPA	85.9% (128/149)
	OPA	91.4 %
Reader B vs. A	PPA	90.9% (130/143)
	NPA	94.4% (117/124),
Reader C vs. A	PPA	81.1% (116/143)
	NPA	98.4% (122/124),
Reader C vs. B	PPA	84.7% (116/137),
	NPA	98.5% (128/130),

Comparison study of *i*VIEW DAB Detection Kit to *ultra*View Universal DAB Detection Kit for HER2-positivity

The Site 1 cohort of 140 FFPE invasive breast carcinoma cases [approximately 35 cases from each HER-2 binning category (0, 1+, 2+, 3+)] was used in a comparison study of *i*VIEW DAB Detection Kit to *ultra*View Universal DAB Detection Kit when staining with PATHWAY anti-HER2 (4B5) antibody on BenchMark ULTRA instrument. A single staining laboratory and three reading sites in the United States participated in the detection comparison study. For PATHWAY anti-HER2 (4B5) antibody staining on the BenchMark ULTRA instrument the PPA rates between results obtained using *i*VIEW DAB Detection Kit and *ultra*View Universal DAB Detection Kit methods based on clinical assessment (positive, negative) were 95.8% (68/71), 96.9% (63/65), and 96.5% (55/57) for Readers A, B, and C, respectively and the NPA rates between detection methods were 90.8% (59/65), 91.5% (65/71), and 97.5% (77/79) for Readers A, B, and C, respectively. The OPA rates between detection kits were 93.4% (127/136), 94.1% (128/136), and 97.1% (132/136) for Readers A, B, and C, respectively. The 3x3 presentation of detection comparison agreement rates for each reader based on clinical score (0/1+, 2+, 3+) are shown in Table 25, Table 26, and Table 27.

Table 25. Reader A, *i*VIEW DAB Detection Kit vs. *ultra*View Universal DAB Detection Kit Agreement Rates 3x3 Analysis – PATHWAY anti-HER2 (4B5) Antibody 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 26. Reader B, *i*VIEW DAB Detection Kit vs. *ultra*View Universal DAB Detection Kit Agreement Rates 3x3 Analysis – PATHWAY anti-HER2 (4B5) Antibody 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 27. Reader C, *i*VIEW DAB Detection Kit vs. *ultra*View Universal DAB Detection Kit Agreement Rates 3x3 Analysis – PATHWAY anti-HER2 (4B5) Antibody 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 for HER2-positivity

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. See Table 28 and Table 29.

Table 28. *i*VIEW DAB Detection Kit Inter-Pathologist Reproducibility Agreement Rates

Comparison	Agreement Rate	% n/N
Reader A vs. B	PPA	100.0% (69/69)
	NPA	92.5% (62/67)
	OPA	96.3%
Reader A vs. C	PPA	98.2% (56/57)
	NPA	77.2% (61/79)
	OPA	86.0%
Reader B vs. C	PPA	96.5% (55/57)
	NPA	82.3% (65/79)
	OPA	88.2%
Reader B vs. A	PPA	93.2% (69/74)
	NPA	100.0% (62/62)
Reader C vs. A	PPA	75.7% (56/74)
	NPA	98.4% (61/62)
Reader C vs. B	PPA	79.7% (55/69)
	NPA	97.0% (65/67)

Table 29. *ultra*View Universal DAB Detection Kit Inter-Pathologist Reproducibility Agreement Rates

Comparison	Agreement Rate	% n/N
Reader A vs. B	PPA	96.9% (63/65)
	NPA	88.7% (63/71)
	OPA	92.6% (126/136)
Reader A vs. C	PPA	98.2% (56/57)
	NPA	81.0% (64/79
	OPA	88.2% (120/136)
Reader B vs. C	PPA	98.2% (56/57)
	NPA	88.6% (70/79)
	OPA	92.6% (126/136)
Reader B vs. A	PPA	88.7% (63/71)
	NPA	96.9% (63/65)
Reader C vs. A	PPA	78.9% (56/71)
	NPA	98.5% (64/65)
Reader C vs. B	PPA	86.2% (56/65)
	NPA	98.6% (70/71)

CLINICAL PERFORMANCE

Comparison Studies of PATHWAY anti-HER2 (4B5) Rabbit Monoclonal Antibody to PATHWAY HER-2 (CB11) Mouse Monoclonal Antibody

A method comparison study was conducted to examine the correlation of PATHWAY anti-HER2 (4B5) antibody to PATHWAY anti-HER-2 (CB11) antibody and PathVysion HER-2 FISH, both previously approved FDA diagnostic tests. Six investigators participated in the study. Two sets of three different investigators evaluated two independent cohorts (Cohort 1: n = 144, Cohort 2: n = 178) using known breast cancer cases stained with HER-2 CB11 and HER2 4B5. FISH data was obtained from patient history. A consensus score from the three readers for each antibody was created for each case to reduce intra-reader variability known to exist with HER-2 scoring.[33,34,35] A total of 322 cases were evaluated. The slides stained with PATHWAY anti-HER-2 (CB11) were processed and stained according to the manufacturer's instructions specified in the VENTANA CB11 method sheet. There was an average of approximately one year between staining and reading of the CB11 stained slides. Since scores from one of the six readers was outside of the confidence interval (CI), data from the two cohorts are presented as follows.

Inter-pathologist Reproducibility of Comparison Studies Specimens

Table 30. Cohort 1: Consensus IHC Scores of Three Pathologists.

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

Cohort 1: Performance characteristics for 3 x 3 Presentation.

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

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

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

Table 31. Cohort 2: Consensus IHC Scores of Three Pathologists.

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

Cohort 2: Performance characteristics for 3 x 3 Presentation.

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

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

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

Table 32. Cohort 1: Consensus CB11 IHC Scores of Three Pathologists Compared to FISH.

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

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

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

Table 33. Cohort 1: Consensus 4B5 IHC Scores of Three Pathologists Compared to FISH.

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

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

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

Table 34. Cohort 2: Consensus CB11 IHC Scores of Three Pathologists Compared to FISH.

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

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

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

Table 35. Cohort 2: Consensus 4B5 IHC Scores of Three Pathologists: Compared to FISH.

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

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

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

Inter-pathologist Reproducibility of Comparison Studies Specimens

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

Table 36. Cohort 1: 4B5 Scoring for the Three Pathologists.

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

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

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

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

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

Table 37. Cohort 1: CB11 Scoring for the Three Pathologists.

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

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

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

Table 38. Cohort 2: 4B5 Scoring for the Three Pathologists.

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

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

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

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

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

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

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

Table 39. Cohort 2: CB11 Scoring for the Three Pathologists.

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

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

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

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

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

Table 40. Ranges of 2X2* Agreements for the Three Pathologists.

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

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

Clinical Outcome Study – KATHERINE

The performance of PATHWAY anti-HER2 (4B5) antibody 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 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 PATHWAY anti-HER2 (4B5) antibody and/or INFORM HER2 Dual ISH assay 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%).

Table 41 describes the overall staining acceptability rate for PATHWAY anti-HER2 (4B5) antibody among the intended to diagnose (ITD) population at the subject level. Out of a total of 1788 subjects in the PATHWAY ITD Population, 55 failed their initial PATHWAY anti-HER2 (4B5) antibody staining attempt. When staining was repeated for these subjects, successful staining was achieved for all but four of them. The initial and final overall staining acceptability rates for the PATHWAY anti-HER2 (4B5) antibody were 96.9% and 99.8%, respectively. The rates of background staining acceptability and morphology acceptability for PATHWAY anti-HER2 (4B5) antibody-stained slides are also reported. Initial and final background staining acceptability rates for the ITD Population were 99.6%, and 99.9%, respectively. Initial and final morphology acceptability rates were 99.2% and 99.9%, respectively.

Table 41. PATHWAY anti-HER2 (4B5) antibody staining performance characteristics.

Attribute	Acceptability rate % (n/N) (95% CI)	
	Initial*	Final**
Overall staining acceptability rate	96.9 (1733/1788) (96.0, 97.6)	99.8 (1784/1788) (99.4, 99.9)
Background	99.6 (1768/1775) (99.2, 99.8)	99.9 (1786/1787) (99.7, 100.0)
Morphology	99.2 (1762/1776) (98.7, 99.5)	99.9 (1787/1788) (99.7, 100.0)

* The initial staining attempt is the first staining attempt for a subject

** The final staining attempt is the staining attempt that was used for enrollment decision in study BO27938

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.

KADCYLA was given intravenously at 3.6 mg/kg on Day 1 of a 21-day cycle. Trastuzumab was given intravenously at 6 mg/kg on Day 1 of a 21-day cycle. Patients were treated with KADCYLA or trastuzumab for a total of 14 cycles unless there was recurrence of disease, withdrawal of consent, or unacceptable toxicity, whichever occurred first. At the time of the primary analysis, median treatment duration was 10 months (range: 1–12) for KADCYLA, and median treatment duration 10 months (range: 1–13) for trastuzumab. Patients who discontinued KADCYLA could complete the duration of their intended study treatment up to 14 cycles of HER2-directed therapy with trastuzumab if appropriate based on toxicity considerations and investigator discretion.

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.

Patient demographics and baseline tumor characteristics were balanced between treatment arms. The median age was approximately 49 years (range 23-80 years), 72.8% were White, 8.7% were Asian and 2.7% were Black or African American. All but 5 patients were women. 22.5 percent of patients were enrolled in North America, 54.2% in Europe and 23.3% throughout the rest of the world. Tumor prognostic characteristics including hormone receptor status (positive: 72.3%, negative: 27.7%), clinical stage at presentation (inoperable: 25.3%, operable: 74.8%) and pathological nodal status after preoperative therapy (node positive: 46.4%, node negative not evaluated: 53.6%) were similar in the study arms.

The majority of the patients (76.9%) had received an anthracycline-containing neoadjuvant chemotherapy regimen. 19.5% of patients received another HER2-targeted agent in addition to trastuzumab as a component of neoadjuvant therapy. Pertuzumab was the second therapy in 93.8% of patients who received a second neoadjuvant HER2-directed agent.

Efficacy results are presented in Table 42 and Figure 2.

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 42. Efficacy results from KATHERINE.

	KADCYLA N= 573	Trastuzumab N= 559
Primary Endpoint	**Invasive Disease Free Survival (IDFS)**[1]	
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[2] %	89.0	75.7

1. Data from first interim analysis
2. 3-year event-free rate derived from Kaplan-Meier estimates

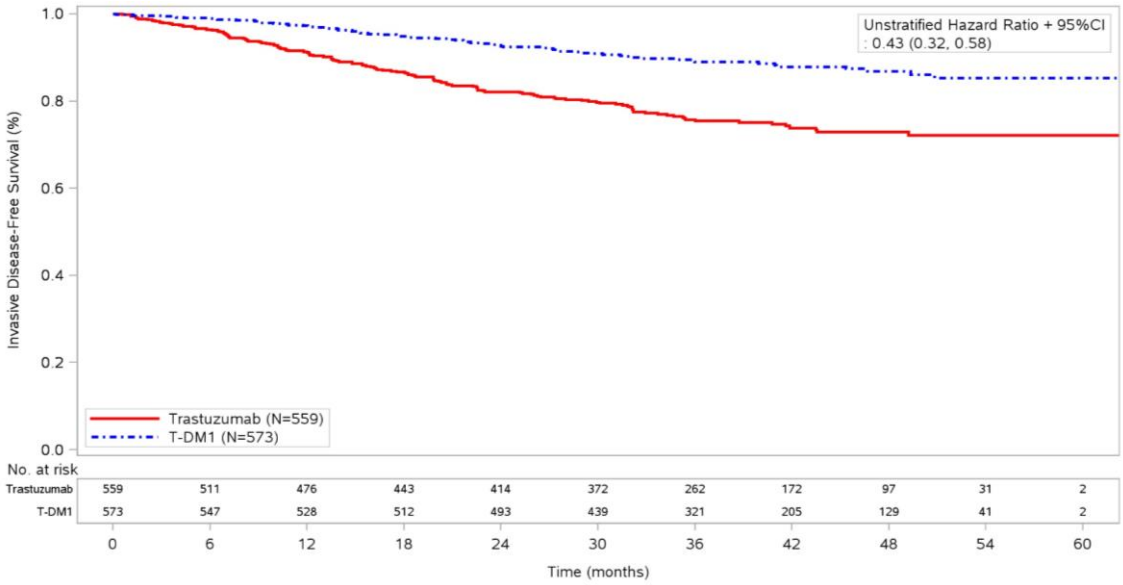

Figure 2. Kaplan-Meier curve of invasive disease free survival in KATHERINE.

Clinical Outcome Study- DESTINY-Breast04

DESTINY-Breast04 was a phase III multicenter, randomized, open-label, active controlled trial evaluating the safety and efficacy of fam-trastuzumab deruxtecan-nxki (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 PATHWAY 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 fam-trastuzumab deruxtecan-nxki (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). Fam-trastuzumab deruxtecan-nxki (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 43).

Table 43. PFS and OS per BIRC in the Hormone Receptor-positive Population and Full Analysis Set (DESTINY-Breast04)

Parameter	Hormone Receptor-positive Population		Full Analysis Set	
	Fam-trastuzumab deruxtecan-nxki (ENHERTU®) N = 331	Treatment of Physician Choice N = 163	Fam-trastuzumab deruxtecan-nxki (ENHERTU®) N = 373	Treatment of Physician Choice N = 184
Median PFS [a], months [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.

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. For corrective action, refer to the Step By Step Procedure section, the instrument User Guide or contact your local support representative.

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