VENTANA®

Roche

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

REF 790-2991

05278368001

IVD Σ 50

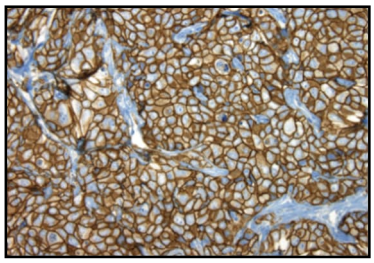

Figure 1. PATHWAY anti-HER2 (4B5) antibody staining in breast carcinoma.

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 breast carcinoma and biliary tract cancer (gallbladder adenocarcinoma, intrahepatic cholangiocarcinoma, and extrahepatic cholangiocarcinoma) tissue using the *ultra*View Universal DAB Detection Kit on a BenchMark ULTRA or BenchMark ULTRA PLUS instrument.

The IHC device is indicated for identifying patients who are eligible for treatment with the following therapies in accordance with the approved therapeutic labeling:

Indication for use	HER2 Score	Therapy
Breast carcinoma	IHC 3+ or IHC 2+/ISH amplified	Herceptin®
Breast carcinoma	IHC 3+ or IHC 2+/ISH amplified	KADCYLA®
Breast carcinoma	IHC 3+ or ISH amplified*	ENHERTU® + PERJETA®
Breast carcinoma	IHC 0 with membrane staining, IHC 1+ or IHC 2+/ISH non-amplified	ENHERTU®
Biliary tract cancer**	IHC 3+	ZIIHERA®

* Per ASCO/CAP guidelines, the use of HER2 ISH as a reflex test is limited to patients with IHC 2+. In the DB09 trial, all patients were tested with HER2 ISH test regardless of IHC status. In DB09 trial, <1% of screened patients (17/1854) had IHC status less than 2+ and were ISH amplified.

** Biliary tract cancer indication is only approved for use with BenchMark ULTRA instrument.

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

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

SUMMARY AND EXPLANATION

The PATHWAY anti-HER-2/neu (4B5) Rabbit Monoclonal Primary Antibody (PATHWAY anti-HER2 (4B5) antibody) is designed to detect the HER2 protein by immunohistochemistry (IHC). The HER2 protein (also known as the c-erbB-2 oncoprotein) is a member of the epidermal growth factor subfamily of transmembrane receptor tyrosine kinases that mediate the growth, differentiation, and survival of cells.[1,2,6] Protein overexpression, due to amplification of the HER2 gene, drives tumorigenesis in breast cancer.[1] Excess HER2 at the cell membrane enhances signal transduction, which upregulates proliferation and differentiation and ultimately causes tumor formation.[1,2,3] The signaling imbalance also confers survival properties to the malignant cell by downregulating apoptotic pathways.[4] Receptor overexpression also leads to upregulated expression of hyperactive HER2 variants produced from alternative translational initiation or proteolytic cleavage (shedding) of the extracellular domain which promote tumor progression and metastasis.[5,6]

Many tumor types, predominantly malignancies of epithelial origin, demonstrate overexpression of the HER2 protein, amplification of the HER2 gene, or both.[7] HER2 is an established biomarker for HER2-targeted therapy patient selection in breast and gastric/gastroesophageal cancer.[3] The association between HER2 expression/amplification and clinical benefit with HER2-targeted therapy has been reported across multiple cancers.

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 or BenchMark ULTRA PLUS 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 or BenchMark ULTRA PLUS 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 or BenchMark ULTRA PLUS instrument, reduces the possibility of human error and inherent variability resulting from individual reagent dilution, manual pipetting, and manual reagent application.

MATERIAL 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 appropriate VENTANA detection kit package insert (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 package insert (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, Superfrost Plus (VWR Cat. No. 48311-703 or equivalent)
3. CONFIRM Negative Control Rabbit Ig (Cat. No. 760-1029 / 05266238001) (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)
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. PATHWAY HER-2 4 in 1 Control Slides (Cat. No. 781-2991 / 05273510001)
12. Permanent mounting medium
13. Cover glass
14. Automated coverslipper
15. General purpose laboratory equipment
16. BenchMark ULTRA or BenchMark ULTRA PLUS 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 ULTRA or BenchMark ULTRA PLUS 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.[8] 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).[8]

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."

Sections should be cut at approximately 4 µm in thickness and mounted on positively charged slides. The slides should be Superfrost Plus or equivalent. Tissue should be air dried by placing the slides at ambient temperature overnight.[8] 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.

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.[9,10.]
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 OSHA criteria for a hazardous substance. Buildup of NaN_3 may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide accumulation in plumbing.[11] 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 table below for the staining protocols. 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 validate 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.

For more details on the proper use of this device, refer to the inline dispenser package insert (method sheet) associated with P/N 790-2991.

The staining protocols and procedures listed in Table 1 are appropriate for use in all HER2 screening of breast carcinoma or biliary tract cancer (BTC) cases as indicated in the table, when used with the associated instrument. Deviating from the recommended staining protocol may produce invalid results, particularly in cases with HER2-low expression (IHC 1+) and HER2-ultralow expression (IHC 0 with membrane staining). Decreasing or increasing cell conditioning times are likely to produce HER2-stained samples with altered HER2 scores, which may result in inappropriate treatment decisions for patients.

Table 1. Staining protocols for PATHWAY anti-HER2 (4B5) antibody for HER2 assessment on a BenchMark instrument, based on indication for use and instrument.

Procedure Type	PATHWAY anti-HER2 (4B5)	PATHWAY anti-HER2 (4B5)
Tissue / Indication(s)	Breast carcinoma	Biliary tract cancer**
Instrument Platform	BenchMark ULTRA & BenchMark ULTRA PLUS	BenchMark ULTRA **
Staining Procedure:	U PATHWAY HER2 4B5	UT PATHWAY HER2 4B5**
Protocol step	Parameter input	Parameter input
Deparaffinization*	Selected, 4 minutes, 72 °C	Selected, 4 minutes, 72 °C
Cell Conditioning*	ULTRA CC1, 36 minutes, Mild (95 °C)	ULTRA CC1, 36 minutes, Mild (95 °C)
Antibody (Primary)*	PATHWAY HER2 4B5 Ab, 12 Min, 36 °C Or Neg Ctl Rbt Ig, 12 Min, 36 °C	PATHWAY HER2 4B5 Ab, 12 Min, 36 °C Or Neg Ctl Rbt Ig, 12 Min, 36 °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 H2O2, 8 minutes, 36 °C *ultra*View Copper, 4 minutes, 36 °C	*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 H2O2, 8 minutes, 36 °C *ultra*View Copper, 4 minutes, 36 °C
Counterstain	Hematoxylin II, 4 minutes, 36 °C	Hematoxylin II, 4 minutes, 36 °C
Post Counterstain	Bluing, 4 minutes, 36 °C	Bluing, 4 minutes, 36 °C

* These are pre-programmed conditions and are not selectable steps to the user.

** UT PATHWAY HER2 4B5: This staining procedure is to be used for BTC samples and can only be installed on the BenchMark ULTRA instrument. As noted in the table for breast cancer samples, the U PATHWAY HER2 4B5 procedure can be installed on both the instruments, BenchMark ULTRA and BenchMark ULTRA PLUS. The prefix "UT" denotes that this procedure must be used for BTC samples on the BenchMark ULTRA instrument. The performance of BTC samples on the BenchMark ULTRA PLUS instrument platform has not been validated and may produce incorrect results.

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

PATHWAY HER-2 4 in 1 Control Slides contains four formalin-fixed cell line controls embedded in paraffin, sectioned and placed on a single charged slide, which 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 package insert (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 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), which may serve as a positive control tissue for either breast carcinoma or BTC patient samples. 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, 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 package insert (method sheet). 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[12]). These quality control procedures should be repeated for each new antibody lot, or whenever there is a change in assay parameters. Breast cancer or biliary tract carcinoma 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 in Breast Carcinoma

Below is a quick reference chart for staining criteria. Refer to PATHWAY anti-HER-2/neu (4B5) Rabbit Monoclonal Primary Antibody Interpretation Guide for Breast Cancer (P/N 14991US) for a more detailed description with photographs of staining with PATHWAY anti-HER2 (4B5) antibody in breast carcinoma.

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

Staining pattern	HER2 (4B5) Score (Report to treating physician)	HER2 Status	Therapy
No membrane staining is observed*	IHC 0 absent membrane staining	HER2-null	None
Any staining of the membrane in greater than 0 and less than or equal to 10% of the cancer cells*,**,***	IHC 0 with membrane staining	HER2-ultralow expression	ENHERTU® (fam-trastuzumab deruxtecan-nxki)
Faint, partial staining of the membrane in greater than 10% of the cancer cells*	IHC 1+	HER2-low expression	
Weak to moderate complete staining of the membrane in greater than 10% of the cancer cells	IHC 2+**** *Reflex test: HER2 Non-Amplified*	HER2-low expression	
	IHC 2+**** *Reflex test: HER2 Amplified*	HER2 positive / overexpression	Herceptin® (trastuzumab) KADCYLA® (trastuzumab emtansine) ENHERTU® (fam-trastuzumab deruxtecan-nxki) + PERJETA® (pertuzumab)*****
Intense complete staining of the membrane in greater than 10% of the cancer cells	IHC 3+	HER2 positive / overexpression	

* Review at 40x is recommended to discern the presence or absence of any staining such as faint, partial staining.

** Recommend re-reading by a second pathologist for cases classified as HER2-null or as HER2-ultralow expression with %Tumor Cells (%TC) ≤ 5%.

*** In the HER2-ultralow "IHC 0 with membrane staining" category, partial membranous staining is usually faint but may exhibit stronger intensities, and such rare cases are scored as HER2-ultralow if they do not otherwise qualify for a higher score. Refer to the Interpretation Guide for case examples.

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

***** Per ASCO/CAP guidelines, the use of HER2 ISH is limited to patients with IHC 2+. In the DB09 trial, all patients were tested for HER2 ISH regardless of IHC status. In that clinical trial, <1% (17/1854) of screened patients had IHC status less than 2+ and ISH amplification.

Scoring Conventions for the Interpretation of PATHWAY anti-HER2 (4B5) Antibody in Biliary Tract Cancer

Below is a quick reference chart for staining criteria. Refer to PATHWAY anti-HER-2/neu (4B5) Rabbit Monoclonal Primary Antibody Interpretation Guide for Biliary Tract Cancer (P/N 23356EN) for a more detailed description with photographs of staining with PATHWAY anti-HER2 (4B5) antibody.

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

Staining pattern (resection specimen)	Staining pattern (biopsy specimen*)	HER2 (4B5) Score (report to requesting physician)	HER2 Status	ZIHERA® (zanidatamab-hrii)
No reactivity or membranous reactivity in < 10% of tumor cells	No reactivity or membranous reactivity in any tumor cell	IHC 0	HER2 Negative	Treatment ineligible
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	IHC 1+		
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***	IHC 2+		
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***	IHC 3+	HER2 Positive	Treatment eligible

* Biopsy specimens include endoscopic, pinch (forceps), and needle core

** ≥ 5 cohesive cells

*** Recommend re-reading by a second pathologist for cases with "strong complete basolateral or lateral membranous reactivity" and a %TC (resection specimens) near the threshold of 10% (range of %TC between 1%-20%) or tumor cell cluster (biopsy specimens) of 5 cohesive cells (tumor cell cluster between 1-20 cohesive cells).

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, correct instrument and staining protocol selection according to the tissue type, 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 incorrect instrument and staining protocol selection, 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. This product is not intended for use in flow cytometry, performance characteristics have not been determined.
6. 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.[13] Contact your local support representative with documented unexpected reactions.
7. Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit nonspecific staining with horseradish peroxidase.[14]
8. 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.[15]
9. 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 stained samples with changed HER2 scores which may cause inappropriate treatment decisions for patients. Increased hematoxylin incubation times are likely to produce PATHWAY anti-HER2 (4B5) antibody stained samples with changed HER2 scores in BTC specimens, 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”.[16]
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 45 days of antigen stability on unstained slides. Because environmental factors are known to affect antigen stability on cut slides, laboratories should validate cut slide stability within their own environment.
4. Immunohistochemical staining with clone 4B5 can produce cytoplasmic and nuclear staining of neoplastic cells in BTC. This staining pattern should not be confused with the discrete membranous staining that is indicative of HER2 positivity in neoplastic cells.
5. All assays might not be registered on every instrument. Please contact your local Roche representative for more information.
6. 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.[17]

PERFORMANCE CHARACTERISTICS

ANALYTICAL PERFORMANCE

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

Sensitivity

The intended use prevalence across HER2 categories has been studied through distribution available in commercial tissue banks for analytical studies as well as through review of the clinical trials. Specimens were stained on a BenchMark ULTRA instrument.

Analytical sensitivity was evaluated by characterizing HER2 distribution (percent) among breast cancer tissue specimens from commercial tissue banks used in the analytical studies, in Table 5 below.

Table 5. Distribution* of HER2 IHC Scores in commercial cohort.

HER2 IHC Bin	n/N	%
IHC 0 absent membrane staining	124 / 408	30.4
IHC 0 with membrane staining	135 / 408	33.1
IHC 1+	40 / 408	9.8
IHC 2+**	24 / 408	5.9
IHC 3+	85 / 408	20.8

* In different populations, prevalence of HER2 IHC scores are different from the distribution presented in this table. Note: The commercial cohort used in this study was enriched and not a random population.

** The IHC 2+ category includes both ISH amplified and non-amplified.

In addition to the analytical studies where samples were sourced commercially, the prevalence was estimated based on clinical trials screening for inclusion / exclusion criteria. Additionally, the scoring algorithm in each clinical trial is a factor in which HER2 IHC Bins are represented in each analysis. Table 5 (above) and Table 6 (below) present the distribution of scores using a modified version of the 2023 ASCO CAP Breast Cancer Guidelines; the scoring algorithm in these analysis include the HER2-ultralow category/bin “IHC 0 with membrane staining” to subdivide the traditionally HER2-negative bin, while Table 7 was from an earlier clinical trial before the modified scoring algorithm was introduced via DESTINY-Breast06 (Table 6). Table 6 and Table 7 provide the prevalence in the DESTINY-Breast06 and DESTINY-Breast04 clinical trials, respectively.

Table 6. Prevalence* of HER2 IHC Scores in Clinical Trial DESTINY-Breast06.

HER2 IHC Bin	n/N	%
IHC 0 absent membrane staining	225/1,940	11.6
IHC 0 with membrane staining	400/1,940	20.6
IHC 1+	828/1,940	42.6
IHC 2+**	423/1,940	21.8
IHC 3+	4/1,940	0.2
Not evaluable	60/1,940	3.1

* In different populations, prevalence of HER2 IHC scores are different from the prevalence presented in this table for the DB06 clinical trial population. Note that HER2-positive (IHC 2+/ISH+ and IHC 3+) is underrepresented in this DB06 clinical trial population because the clinical trial design only included participants with a history of HER2-low or negative expression, defined as IHC 2+/ISH- or IHC 1+ (ISH – or untested) or IHC 0 (ISH- or untested) with a validated assay. American Cancer Society (ACS) and CAP ASCO have noted that HER2 positive may be 15-20% in the overall breast cancer population.

** The IHC 2+ category includes both ISH amplified and non-amplified.

Analytical sensitivity in Table 7 was evaluated by characterizing HER2 prevalence (percent) among breast cancer tissue specimens in clinical trial DESTINY-Breast04.

Table 7. Prevalence* of HER2 IHC Scores in Clinical Trial DESTINY-Breast04.

HER2 IHC Bin	n/N	%
IHC 0	267/1,303	20.5
IHC 1+	554/1,303	42.5
IHC 2+	440/1,303	33.8
IHC 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 this table. Note that HER2-positive (IHC 2+/ISH+ and IHC 3+) is underrepresented in this DB04 clinical trial population because the study design only included participants with a history of low HER2 expression defined as IHC 2+/ISH- or IHC 1+ (ISH- or untested)

The DESTINY-Breast04 clinical trial utilized the 2018 ASCO CAP Breast Cancer Guidelines, and therefore did not include the HER2-ultralow scoring category (IHC 0 with membrane staining), since that was introduced later, following the DESTINY-Breast06 clinical trial (Table 6).

Analytical sensitivity for biliary tract cancer was evaluated by characterizing HER2 prevalence (percent) among BTC specimens in the clinical trial HERIZON-BTC-01.

Table 8. Prevalence* of HER2 IHC Scores in Clinical Trial HERIZON-BTC-01.

HER2 IHC Bin	n/N	%
IHC 0	353/806	43.8
IHC 1+	108/806	13.4
IHC 2+	205/806	25.4
IHC 3+	112/806	13.9
Not evaluable	28/806	3.5

* In different populations, prevalence of HER2 IHC scores can be different from the prevalence presented in Table 6.

Specificity

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 9 and Table 10. The study showed no specific membrane staining for most normal tissues. Specimens were stained on a BenchMark ULTRA instrument.

Positive staining in tonsilar epithelium, bladder, 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.

Table 9. 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

Table 10. Specificity 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

Pathology	# Positive / Total Cases
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
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

Pathology	# Positive / Total Cases
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
Adenocarcinoma (Gallbladder)	33/100
Cholangiocarcinoma (intra- and extra-hepatic)	19/100

Precision - HER2-ultralow Breast Cancer

For an evaluation of the precision of the PATHWAY anti-HER2 (4B5) antibody on BenchMark ULTRA instrument, 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-ultralow on BenchMark ULTRA Instrument

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 (between antibody kit lot)
- Three lots of *ultra*View Universal 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)
- 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-ultralow therapy. Among 24 cases, there were 6 cases with majority HER2 bin of IHC 0 absent membrane staining, 6 cases with majority HER2 bin of IHC 0 with membrane staining (IHC >0 <1+), 3 cases with majority HER2 bin of IHC 1+, 3 cases with majority HER2 bin of IHC 2+ and 6 cases with majority HER2 bin of IHC 3+. Results of this analysis are presented in Table 11 and Table 12.

Table 11. Median and Range of %TC for Cases in the Intermediate Precision Study for HER2-ultralow on BenchMark ULTRA Instrument.

Case	Majority HER2 IHC Bin	Median %TC	Range %TC (Min-Max)	Percent Eligible
1	0	0.0	0 - 0	0.0% (0/18)
2	0	0.0	0 - 0	0.0% (0/18)
3	0	0.0	0 - 0	0.0% (0/18)
4	0	0.0	0 - 0	0.0% (0/18)
5	0	0.0	0 - 0	0.0% (0/18)
6	0	0.0	0 - 0	0.0% (0/18)
7	>0<1+	2.0	2 - 5	100.0% (18/18)
8	>0<1+	5.0	2 - 5	100.0% (18/18)
9	>0<1+	5.0	5 - 10	100.0% (18/18)
10	>0<1+	5.0	5 - 10	100.0% (18/18)
11	>0<1+	10.0	5 - 10	100.0% (18/18)
12	>0<1+	10.0	5 - 10	100.0% (18/18)
13	1+	20.0	15 - 20	100.0% (18/18)
14	1+	20.0	15 - 30	100.0% (17/17)
15	1+	40.0	30 - 40	100.0% (3/3)
16	2+	15.0	15 - 15	100.0% (18/18)
17	2+	20.0	15 - 70	100.0% (18/18)
18	2+	60.0	50 - 60	100.0% (18/18)
19	3+	20.0	15 - 29	0.0% (0/18)
20	3+	80.0	70 - 80	0.0% (0/18)
21	3+	80.0	80 - 80	0.0% (0/18)
22	3+	90.0	80 - 90	0.0% (0/18)
23	3+	90.0	90 - 90	0.0% (0/18)
24	3+	100.0	90 - 100	0.0% (0/18)

Twenty four (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 24 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 Table 12.

Table 12. Precision Components for Cases in Intermediate Precision Study for HER2-ultralow on BenchMark ULTRA Instrument.

Case	Majority HER2 IHC 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	0.00	0.00	0.00
4	0	15.0	0.00	0.00	0.00	0.00	0.00	0.00
5	0	15.0	0.00	0.00	0.00	0.00	0.00	0.00
6	0	17.5	0.00	0.00	0.00	0.00	0.00	0.00
7	>0<1+	20.0	0.00	0.00	1.73	1.73	1.73	3.00
8	>0<1+	20.0	0.00	1.73	0.00	0.00	0.00	1.73
9	>0<1+	20.0	0.00	2.89	0.00	0.00	0.00	2.89
10	>0<1+	22.5	0.00	2.89	0.00	0.00	0.00	2.89
11	>0<1+	25.0	2.04	2.50	0.00	0.00	0.00	3.23
12	>0<1+	30.0	0.00	0.00	2.89	2.89	2.89	5.00
13	1+	50.0	0.00	2.89	0.00	0.00	0.00	2.89
14	1+	20.0	0.00	5.77	6.45	0.00	6.45	10.80
15	1+	20.0	0.00	7.07	0.00	0.00	0.00	7.07
16	2+	25.0	0.00	0.00	0.00	0.00	0.00	0.00
17	2+	35.0	0.00	2.89	28.72	0.00	5.00	29.30
18	2+	35.0	0.00	0.00	0.00	0.00	5.77	5.77
19	3+	50.0	2.12	0.00	2.12	2.47	0.00	3.88
20	3+	60.0	0.00	0.00	0.00	5.77	0.00	5.77
21	3+	75.0	0.00	0.00	0.00	0.00	0.00	0.00
22	3+	95.0	0.00	0.00	5.77	5.77	0.00	8.16
23	3+	100.0	0.00	0.00	0.00	0.00	0.00	0.00
24	3+	100.0	3.33	1.67	0.00	0.00	0.00	3.73

In addition, a qualitative analysis of different precision components was performed for HER2-ultralow on BenchMark ULTRA instrument. For the purposes of study analysis, HER2 scores “IHC 0 absent membrane staining“ and “IHC 3+” were grouped together as negative cases because they were ineligible for HER2-ultralow therapy per the clinical trial design, and HER2 scores of “IHC 0 with membrane staining”, “IHC 1+” and “IHC 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 13.

Table 13. Repeatability and Intermediate Precision of PATHWAY anti-HER2 (4B5) antibody on Breast Cancer Tissues with HER2-ultralow scoring on BenchMark ULTRA Instrument.

Repeatability / Precision	Agreement			
	Type	n/N	%	95% CI
Between-Antibody Lots	PPA	67/67	100.0	(94.6, 100.0)
	NPA	72/72	100.0	(94.9, 100.0)
	OPA	139/139	100.0	(97.3, 100.0)
Between-Detection Kits	PPA	67/67	100.0	(94.6, 100.0)
	NPA	72/72	100.0	(94.9, 100.0)
	OPA	139/139	100.0	(97.3, 100.0)
Between-Instruments (BenchMark ULTRA)	PPA	67/67	100.0	(94.6, 100.0)
	NPA	72/72	100.0	(94.9, 100.0)
	OPA	139/139	100.0	(97.3, 100.0)
Between-Day	PPA	68/68	100.0	(94.7, 100.0)
	NPA	72/72	100.0	(94.9, 100.0)
	OPA	140/140	100.0	(97.3, 100.0)
Within-Run	PPA	99/99	100.0	(96.3, 100.0)
	NPA	108/108	100.0	(96.6, 100.0)
	OPA	207/207	100.0	(98.2, 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. CIs for 100% PPA, NPA and OPA were calculated using Wilson score method.

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

Reader Precision for HER2-ultralow on BenchMark ULTRA Instrument

Between-Reader and Within-Reader precision was assessed by evaluating concordance of HER2 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 status per Pattern of Cell Membrane Staining with PATHWAY anti-HER2 (4B5) antibody staining (Table 3). 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 Table 14 and Table 15.

Table 14. Results of the Reader Precision Study for HER2-ultralow on BenchMark ULTRA Instrument.

Case Category	HER2 IHC	N of Cases	N of Reads	Results by HER2 IHC, %TC Category				
				IHC 0, No Staining	IHC >0 <1+, Faint Incomplete ≤ 10%	IHC 1+, Faint Incomplete > 10%	IHC 2+, Weak to Moderate Complete	IHC 3+, Intense Complete
No staining	0	32	192	192	0	0	0	0
No staining/faint incomplete ≤ 10%	0/>0<1+	3	18	9	9	0	0	0
Faint incomplete ≤ 10%	>0<1+	9	54	0	54	0	0	0
Faint incomplete ≤ 10% / > 10%	>0<1+/1+	3	18	0	15	3	0	0
Faint incomplete ≤ 10% / > 10%	>0<1+/1+	4	24	0	12	12	0	0
Faint incomplete > 10%	1+	11	66	0	0	66	0	0
Faint incomplete > 10%/weak to moderate complete	1+/2+	4	24	0	0	18	6	0
Faint incomplete > 10% / weak to moderate complete	1+/2+	3	18	0	0	5	13	0
Weak to moderate complete	2+	14	84	0	0	0	84	0
Weak to moderate complete / Intense complete	2+/3+	5	30	0	0	0	12	18
Very variable	0/3+	1	6	1	0	0	0	5
Intense complete	3+	11	66	0	0	0	0	66

Table 15. Precision Components for Cases in Reader Precision Study for HER2-ultralow on BenchMark ULTRA Instrument.

Case Category	HER2 IHC	N of Cases	Range of Median %TC	SD			Percent Results "Eligible"
				Within-Reader	Between-Reader	Total	
No staining	0	32	0.0 - 0.0	0.0	0.0	0.0	0.0% (0/192)
No staining/faint incomplete ≤ 10%	0/>0<1+	3	0.0 - 1.0	0.5	0.4	0.5	50.0% (9/18)
Faint incomplete ≤ 10%	>0<1+	8	2.0 - 9.5	1.4	1.4	1.4	100.0% (54/54)
Faint incomplete ≤ 10% / > 10%	>0<1+/1+	3	5.0 - 10.0	2.9	0.0	2.9	100.0% (18/18)
Faint incomplete ≤ 10% / > 10%	>0<1+/1+	4	8.0 - 12.5	6.0	3.9	6.0	100.0% (24/24)
Faint incomplete > 10%	1+	11	15.0 - 55.0	14.9	5.1	14.9	100.0% (66/66)
Faint incomplete > 10% / weak to moderate complete	1+/2+	4	N/A	N/A	N/A	N/A	100.0% (24/24)
Faint incomplete > 10% / weak to moderate complete	1+/2+	3	N/A	N/A	N/A	N/A	100.0% (18/18)
Weak to moderate complete	2+	14	15.0 - 77.5	13.7	16.6	13.7	100.0% (84/84)
Weak to moderate complete / Intense complete	2+/3+	5	N/A	N/A	N/A	N/A	40.0% (12/30)
Very variable	0/3+	1	N/A	N/A	N/A	N/A	0.0% (0/6)
Intense complete	3+	11	30.0 - 100.0	8.2	16.3	8.2	0.0% (0/66)

In addition, a qualitative analysis of different precision components was performed. For the purposes of study analysis, HER2 scores "IHC 0 absent membrane staining" and "IHC 3+" were grouped together as negative cases because they were ineligible for HER2-ultralow therapy per the clinical trial design, and HER2 scores of "IHC 0 with membrane staining", "IHC 1+" and "IHC 2+" were grouped together as positive cases as they were eligible or potentially eligible for HER2-ultralow targeted therapy per the trial design. The agreement for between-reader and within-reader precision are summarized in Table 16.

Table 16. Within and Between-Reader Precision of the PATHWAY anti-HER2 (4B5) antibody with HER2-ultralow scoring on BenchMark ULTRA Instrument.

Precision	Agreement			
	Type	n/N	%	95% CI
Within-Reader	APA	302/309	97.7	(95.9, 99.3)
	ANA	284/291	97.6	(95.6, 99.3)
	OPA	293/300	97.7	(95.7, 99.3)
Between-Reader	APA	296/310	95.5	(91.7, 98.4)
	ANA	276/290	95.2	(91.1, 98.2)
	OPA	286/300	95.3	(92.0, 98.0)

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.

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

Inter-laboratory Reproducibility Study for HER2-ultralow on BenchMark ULTRA Instrument

An Inter-Laboratory Reproducibility Study of the PATHWAY anti-HER2 (4B5) antibody was completed to demonstrate reproducibility of the assay to determine HER2-ultralow 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-ultralow 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-ultralow therapy. Results of this analysis for each case were presented in Table 17.

Table 17. Results of the Inter-Laboratory Reproducibility Study for HER2-ultralow on BenchMark ULTRA Instrument.

Case	Majority HER2 IHC Bin	N of Reads	IHC 0, No Staining	IHC 0, Faint Incomplete ≤ 10%	IHC 1+, Faint Incomplete > 10%	IHC 2+, Weak to Moderate Complete	IHC 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	100% (30/30)	0	0	0	0	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
4	0	30	100% (30/30)	0	0	0	0	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
5	0	30	100% (30/30)	0	0	0	0	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
6	0	30	100% (30/30)	0	0	0	0	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
7	0	30	100% (30/30)	0	0	0	0	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
8	>0<1+	30	3% (1/30)	93% (28/30)	3% (1/30)	0	0	90% (9/10)	100% (10/10)	100% (10/10)	97% (29/30)
9	>0<1+	30	10% (3/30)	87% (26/30)	3% (1/30)	0	0	90% (9/10)	100% (10/10)	80% (8/10)	90% (27/30)
10	>0<1+	30	0	63% (19/30)	37% (11/30)	0	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)
11	>0<1+	30	0	87% (26/30)	13% (4/30)	0	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)
12	>0<1+	30	0	57% (17/30)	30% (9/30)	13% (4/30)	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)
13	>0<1+	30	0	53% (16/30)	47% (14/30)	0	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)
14	1+	30	0	13% (4/30)	87% (26/30)	0	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)
15	1+	30	0	0	83% (25/30)	17% (5/30)	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)
16	1+	30	0	0	67% (20/30)	33% (10/30)	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)
17	1+	30	0	0	83% (25/30)	17% (5/30)	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)
18	2+	30	0	0	0	100% (30/30)	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)
19	2+	30	0	0	0	100% (30/30)	0	100% (10/10)	100% (10/10)	100% (10/10)	100% (30/30)
20	2+	30	0	0	0	93% (28/30)	7% (2/30)	80% (8/10)	100% (10/10)	100% (10/10)	93% (28/30)
21	2+	30	0	0	0	97% (29/30)	3% (1/30)	90% (9/10)	100% (10/10)	100% (10/10)	97% (29/30)
22	3+	30	0	0	0	0	100% (30/30)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)

Case	Majority HER2 IHC Bin	N of Reads	IHC 0, No Staining	IHC 0, Faint Incomplete ≤ 10%	IHC 1+, Faint Incomplete > 10%	IHC 2+, Weak to Moderate Complete	IHC 3+, Intense Complete	Percent Results "Eligible"			
								Site A	Site B	Site C	Overall
23	3+	30	0	0	0	0	100% (30/30)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
24	3+	30	0	0	0	0	100% (30/30)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
25	3+	30	0	0	0	0	100% (30/30)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
26	3+	30	0	0	0	0	100% (30/30)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/30)
27	3+	30	3% (1/30)	0	0	0	97% (29/30)	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)

Twenty one (21) out of 28 cases had 30 results with the same type of staining ("No staining" or "Faint, partial staining ≤ 10%," "Faint, partial staining > 10%," 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: between-reader, between-day, between-site and total. Results are summarized in Table 18.

Table 18. Precision Components for Cases in the Inter-Laboratory Reproducibility Study for HER2-ultralow on BenchMark ULTRA Instrument.

Case	Case category	HER2 IHC 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
3	No staining	0	30	0.0	0-0	0.0	0.0	0.0	0.0
4	No staining	0	30	0.0	0-0	0.0	0.0	0.0	0.0
5	No staining	0	30	0.0	0-0	0.0	0.0	0.0	0.0
6	No staining	0	30	0.0	0-0	0.0	0.0	0.0	0.0
7	No staining	0	30	0.0	0-0	0.0	0.0	0.0	0.0
8	Faint incomplete ≤ 10%	>0<1+	30	1.0	0 - 20	2.4	0.7	0.0	3.9
9	Faint incomplete ≤ 10%	>0<1+	30	2.5	0 - 15	3.1	1.1	0.0	3.8
10	Faint incomplete ≤ 10%	>0<1+	30	4.0	1 - 75	4.2	12.1	10.2	19.8
11	Faint incomplete ≤ 10%	>0<1+	30	5.0	1 - 25	5.0	1.9	0.0	6.2
13	Faint incomplete ≤ 10%	>0<1+	30	9.5	1 - 60	13.4	6.6	11.2	19.6
14	Faint incomplete > 10%	1+	30	15.0	1 - 80	0.0	13.9	18.2	27.1
18	Weak to moderate complete	2+	30	45.0	20 - 95	6.5	8.9	14.4	22.4
19	Weak to moderate complete	2+	30	62.5	40 - 90	7.6	0.0	3.8	13.3
22	Intense complete	3+	30	95.0	70 - 100	2.7	4.3	5.7	8.2
23	Intense complete	3+	30	96.5	70 - 100	3.1	2.8	4.6	7.1
24	Intense complete	3+	30	96.5	90 - 100	1.7	0.0	2.6	3.9
25	Intense complete	3+	30	98.0	65 - 100	5.3	3.5	10.6	12.8
26	Intense complete	3+	30	98.0	70 - 100	3.6	2.9	6.9	8.8
28	Intense complete	3+	30	99.0	80 - 100	4.5	1.1	0.0	5.3

The summary of the agreement rates across all evaluable observations, using the sample-level reader modes for HER2-ultralow status as the reference can be found in Table 19.

Table 19. Inter-Laboratory Reproducibility for overall agreement rates for PATHWAY anti-HER2 (4B5) antibody with HER2-ultralow scoring on BenchMark ULTRA Instrument.

Inter-Laboratory Reproducibility	Agreement			
	Type	n/N	%	95% CI
Overall	PPA	413/420	98.3	(96.7, 99.8)
	NPA	420/420	100.0	(99.1, 100.0)
	OPA	833/840	99.2	(98.3, 99.9)
Within-Site	PPA	413/420	98.3	(96.7, 99.8)
	NPA	420/420	100.0	(99.1, 100.0)
	OPA	833/840	99.2	(98.3, 99.9)
Within-Reader	PPA	413/420	98.3	(96.7, 99.8)
	NPA	420/420	100.0	(99.1, 100.0)
	OPA	833/840	99.2	(98.3, 99.9)

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.

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

Table 20. Inter-Laboratory Reproducibility Pairwise Agreement Rates for the PATHWAY anti-HER2 (4B5) antibody with HER2-ultralow scoring on BenchMark ULTRA Instrument.

Inter-Laboratory Reproducibility	Agreement			
	Type	n/N	%	95% CI
Between-Site	APA	8124/8260	98.4	(96.7, 99.8)
	ANA	8404/8540	98.4	(96.9, 99.8)
	OPA	8264/8400	98.4	(96.8, 99.8)
Between-Reader	APA	406/413	98.3	(96.6, 99.8)
	ANA	420/427	98.4	(96.8, 99.8)
	OPA	413/420	98.3	(96.7, 99.8)
Between-Day	APA	1628/1652	98.5	(97.2, 99.8)
	ANA	1684/1708	98.6	(97.4, 99.8)
	OPA	1656/1680	98.6	(97.3, 99.8)

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

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

Concordance Between BenchMark ULTRA PLUS and BenchMark ULTRA Instruments for HER2-ultralow Breast Cancer

Three external laboratories participated in a concordance study between the BenchMark ULTRA PLUS instrument and the BenchMark ULTRA instrument. Tissue slides from 160 breast cancer cases (80 positive and 80 negative, including 16 borderline) were stained with PATHWAY anti-HER2 (4B5) antibody and a negative control internally at Roche Tissue Diagnostics (RTD) on a BenchMark ULTRA instrument using the recommended staining protocol (U PATHWAY HER2 4B5). The ULTRA stained slides were read by one RTD reader and a total of 4 external readers at the 3 laboratories to determine the HER-2 ultralow status (negative: HER2 scores of IHC 0 absent membrane staining and 3+ or positive: HER2 scores of IHC 0 with membrane staining, 1+ and 2+) of these cases. For data analysis each external ULTRA result was matched with the RTD ULTRA result to form 4 scoring categories as shown in Table 21.

Unstained tissue slides from the 160 cases were randomized and equally distributed to the external laboratories for staining on a BenchMark ULTRA PLUS instrument using the recommended staining protocol (U PATHWAY HER2 4B5). One or two readers per site read all the BenchMark ULTRA PLUS slides and determined HER2-ultralow status. All external site readers were blinded to the HER2-ultralow status from the ULTRA-stained slides. ULTRA PLUS results from external site reader(s) were compared to case-matched ULTRA results (RTD-external reader combined result). Results excluding IHC score 3+ are summarized in Table 21.

Table 21. Agreement of HER2-ultralow status for Cases Stained with PATHWAY anti-HER2 (4B5) antibody on the BenchMark ULTRA PLUS versus BenchMark ULTRA Instrument.

BenchMark ULTRA PLUS	BenchMark ULTRA				
	RTD Reader= Positive, External Reader= Positive	RTD Reader= Positive, External Reader= Negative	RTD Reader= Negative, External Reader= Positive	RTD Reader= Negative, External Reader= Negative	Total
Positive	290	7	0	2	299
Negative	13	20	1	133	167
Total	303	27	1	135	466
Percent Positive % (n/N)	95.7 (290/303)	25.9 (7/27)	0.0 (0/1)	1.5 (2/135)	N/A

	n/N	% (95% CI)
PPA	297/330	90.0 (85.5, 94.7)
NPA	134/136	98.5 (96.3, 100.0)

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

Note: Two-sided 95% CI calculated using the percentile bootstrap method.

Note: This table is describing concordance of ULTRA PLUS and ULTRA with regard to eligibility to HER2 targeted therapy in the HER2 ultralow population where HER2 positive is an IHC score of IHC 0 with membrane staining, 1+ or 2+ and negative is IHC null.

Note: Negative does not include 3+ in this table.

Inter-Laboratory Reproducibility Study for HER2-ultralow Breast Cancer on BenchMark ULTRA PLUS

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

Each set of 5 stained slides per sample over 5 staining days was randomized and evaluated by a total of 6 readers (2 readers/ site) for a HER2-ultralow status. Each case had 10 results per site (30 results in total).

The HER2-ultralow 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-ultralow status. The summary of the agreement rates across all evaluable observations, using the sample-level reader modes for HER2-ultralow status as the reference is presented in Table 22. In addition, percent Eligible was calculated with regard to HER2-ultralow therapy.

Table 22. Results of the Inter-Laboratory Reproducibility Study for HER2-ultralow on BenchMark ULTRA PLUS Instrument.

Case	Majority HER2 IHC Bin[b]	N of Reads	IHC 0, No Staining	IHC 0, Faint Incomplete ≤ 10%	IHC 1+, Faint Incomplete > 10%	IHC 2+, Weak To Moderate Complete	IHC 3+, Intense Complete	Percent Results "Eligible"[a]			
								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	97 (29/30)	3 (1/30)	0	0	0	0 (0/10)	10 (1/10)	0 (0/10)	3 (1/30)
3	0	30	93 (28/30)	7 (2/30)	0	0	0	0 (0/10)	10 (1/10)	10 (1/10)	7 (2/30)
4	0	30	100 (30/30)	0	0	0	0	0 (0/10)	0 (0/10)	0 (0/10)	0 (0/30)
5	0	30	100 (30/30)	0	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)	10 (1/10)	0 (0/10)	3 (1/30)
7	0	30	93 (28/30)	7 (2/30)	0	0	0	0 (0/10)	0 (0/10)	20 (2/10)	7 (2/30)
8	>0<1+	30	7 (2/30)	60 (18/30)	33 (10/30)	0	0	80 (8/10)	100 (10/10)	100 (10/10)	93 (28/30)
9	>0<1+	30	7 (2/30)	60 (18/30)	33 (10/30)	0	0	80 (8/10)	100 (10/10)	100 (10/10)	93 (28/30)
10	>0<1+	30	17 (5/30)	83 (25/30)	0	0	0	70 (7/10)	100 (10/10)	80 (8/10)	83 (25/30)
11	1+	30	0	0	77 (23/30)	23 (7/30)	0	100 (10/10)	100 (10/10)	100 (10/10)	100 (30/30)
12	1+	30	0	37 (11/30)	63 (19/30)	0	0	100 (10/10)	100 (10/10)	100 (10/10)	100 (30/30)
13	1+	30	0	23 (7/30)	73 (22/30)	3 (1/30)	0	100 (10/10)	100 (10/10)	100 (10/10)	100 (30/30)
14	1+	30	0	0	70 (21/30)	30 (9/30)	0	100 (10/10)	100 (10/10)	100 (10/10)	100 (30/30)
15	1+	30	0	0	83 (25/30)	13 (4/30)	3 (1/30)	100 (10/10)	100 (10/10)	90 (9/10)	97 (29/30)
16	1+	30	0	7 (2/30)	80 (24/30)	13 (4/30)	0	100 (10/10)	100 (10/10)	100 (10/10)	100 (30/30)
17	2+	30	0	0	3 (1/30)	97 (29/30)	0	100 (10/10)	100 (10/10)	100 (10/10)	100 (30/30)
18	2+	30	0	0	40 (12/30)	60 (18/30)	0	100 (10/10)	100 (10/10)	100 (10/10)	100 (30/30)
19	2+	30	3 (1/30)	0	7 (2/30)	90 (27/30)	0	100 (10/10)	100 (10/10)	90 (9/10)	97 (29/30)
20	2+	30	0	0	3 (1/30)	97 (29/30)	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	3+	30	0	0	0	0	100 (30/30)	0 (0/10)	0 (0/10)	0 (0/10)	0 (0/30)
23	3+	30	0	0	0	0	100 (30/30)	0 (0/10)	0 (0/10)	0 (0/10)	0 (0/30)
24	3+	30	0	0	0	17 (5/30)	83 (25/30)	0 (0/10)	30 (3/10)	20 (2/10)	17 (5/30)
25	3+	30	0	0	0	0	100 (30/30)	0 (0/10)	0 (0/10)	0 (0/10)	0 (0/30)
26	3+	30	0	0	0	0	100 (30/30)	0 (0/10)	0 (0/10)	0 (0/10)	0 (0/30)
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)

[a] Eligible = A HER2 score of 0 with faint incomplete staining, 1+ or 2+.

[b] Majority HER2 IHC bin is the most frequent HER2 IHC bin for each case based on the combined study readers' results.

For each case, a median %TC, range of %TC of 30 results were calculated and SD of between-reader, between-day and between-site are presented in Table 23.

Table 23. Precision Components for Cases in the Inter-Laboratory Reproducibility Study for HER2-ultralow on BenchMark ULTRA PLUS Instrument.

Case	Case Category	HER2 IHC Bin[a]	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 - 1	0.0	0.0	0.0	0.1
3	No staining	0	30	0.0	0 - 1	0.0	0.0	0.0	0.1
4	No staining	0	30	0.0	0 - 0	0.0	0.0	0.0	0.0
5	No staining	0	30	0.0	0 - 0	0.0	0.0	0.0	0.0
6	No staining	0	30	0.0	0 - 1	0.0	0.0	0.0	0.1
7	No staining	0	30	0.0	0 - 3	0.2	0.0	0.0	0.6
8	Faint incomplete ≤10%	>0<1+	30	1.0	0 - 8	1.6	0.2	0.0	2.3
9	Faint incomplete ≤10%	>0<1+	30	5.0	0 - 30	5.9	3.3	0.0	8.9
10	Faint incomplete ≤10%	>0<1+	30	8.0	0 - 20	0.0	3.9	0.0	5.1
11	Faint incomplete >10%	1+	30	11.0	1 - 60	0.0	0.0	3.6	14.1
12	Faint incomplete >10%	1+	30	13.5	2 - 70	2.6	11.1	0.0	16.0
13	Faint incomplete >10%	1+	30	15.0	3 - 60	8.5	4.0	0.0	14.4
14	Faint incomplete >10%	1+	30	16.5	11 - 70	10.9	4.4	0.0	14.9
15	Faint incomplete >10%	1+	30	40.0	20 - 80	8.5	0.0	5.8	16.7
16	Faint incomplete >10%	1+	30	42.0	12 - 95	13.0	0.0	9.6	26.5
17	Weak to moderate complete	2+	30	30.0	11 - 64	11.9	0.0	0.0	20.9
18	Weak to moderate complete	2+	30	34.0	0 - 90	27.9	0.0	0.0	37.0
19	Weak to moderate complete	2+	30	50.0	11 - 90	23.3	15.5	0.0	30.9
20	Weak to moderate complete	2+	30	60.0	12 - 95	15.2	0.0	6.4	26.4
21	Weak to moderate complete	2+	30	60.0	30 - 95	17.1	0.7	0.0	22.7
22	Intense complete	3+	30	85.0	15 - 100	7.4	6.2	0.0	16.4
23	Intense complete	3+	30	90.0	50 - 100	6.3	5.3	7.6	12.5
24	Intense complete	3+	30	90.0	82 - 100	3.2	1.6	2.4	5.5
25	Intense complete	3+	30	95.0	60 - 100	2.3	0.0	1.8	11.5
26	Intense complete	3+	30	95.0	80 - 100	1.1	1.2	4.4	5.9
27	Intense complete	3+	30	99.0	90 - 100	2.1	0.0	0.4	2.7
28	Intense complete	3+	30	99.0	80 - 100	2.3	1.3	1.3	4.2

[a] HER2 IHC bin is based on the majority status of available reads for that case.

Table 24. Inter-laboratory reproducibility for overall agreement rates for PATHWAY anti-HER2 (4B5) antibody with HER2-ultralow scoring in breast carcinoma on the BenchMark ULTRA PLUS instrument.

Inter-Laboratory Reproducibility	Agreement			
	Type	n/N	%	95% CI
Primary Analysis/Overall	PPA	409/420	97.4	(95.5, 99.0)
	NPA	409/420	97.4	(94.8, 99.3)
	OPA	818/840	97.4	(95.8, 98.7)
Site- Stratified	PPA	409/420	97.4	(95.5, 99.0)
	NPA	409/420	97.4	(94.8, 99.3)
	OPA	818/840	97.4	(95.8, 98.7)
Reader-Stratified	PPA	412/425	96.9	(95.0, 98.6)
	NPA	407/415	98.1	(96.5, 99.3)
	OPA	819/840	97.5	(96.1, 98.7)

Note: Two-sided 95% CI calculated using the percentile bootstrap method.

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

In addition, pairwise comparisons were made Between-Site, Between-Reader and Between-Day for HER2-ultralow status. A summary of the results can be found in Table 25.

Table 25. Inter-laboratory reproducibility pairwise agreement rates for PATHWAY anti-HER2 (4B5) antibody with HER2-ultralow scoring in breast carcinoma on the BenchMark ULTRA PLUS instrument.

Inter-Laboratory Reproducibility	Agreement			
	Type	n/N	%	95% CI
Between-Site	APA	7986/8400	95.1	(92.4, 97.4
	ANA	7986/8400	95.1	(92.1, 97.4)
	OPA	7986/8400	95.1	(92.2, 97.4)
Between-Reader	APA	398/420	94.8	(91.7, 97.4)
	ANA	398/420	94.8	(91.4, 97.4
	OPA	398/420	94.8	(91.7, 97.4)
Between-Day	APA	1610/1680	95.8	(93.6, 97.8)
	ANA	1610/1680	95.8	(93.5, 97.8)
	OPA	1610/1680	95.8	(93.6, 97.7)

Note: Two-sided 95% CI calculated using the percentile bootstrap method.

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

Precision - HER2-low Breast Cancer

Intermediate Precision for HER2-low on BenchMark ULTRA Instrument

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 (between-antibody kit lot)
- Three lots of *ultra*View Universal 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)
- One pathologist, 2 replicates

All slides were blinded and randomized prior to evaluation. Samples were evaluated using the criteria for intensity and pattern of cell membrane staining for the scoring algorithm for IHC 0, IHC 1+, IHC 2+, and IHC 3+. Note: The HER2-ultralow category, which subdivided the previous IHC 0 category into IHC 0 absent membrane staining and IHC 0 with membrane staining, was not part of the scoring algorithm at the time of this analysis. See Precision - HER2-ultralow Breast Cancer section for the analysis which used the scoring algorithm in Table 3.

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 IHC 0, 10 cases with majority HER2 bin of IHC 1+, 6 cases with majority HER2 bin of IHC 2+ and 5 cases with majority HER2 bin of IHC 3+. Results of this analysis are presented in Table 26 and Table 27 below.

Table 26. Median and Range of %TC for Cases in the Intermediate Precision Study for HER2-low on BenchMark ULTRA Instrument.

Case	Majority HER2 IHC 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 Table 27.

Table 27. Precision Components for Cases in Intermediate Precision Study for HER2-low on BenchMark ULTRA Instrument.

Case	Majority HER2 IHC 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 HER2-low on BenchMark ULTRA instrument. For the purposes of study analysis, HER2 scores “IHC 0“ and “IHC 3+” were grouped together as negative cases because they were ineligible for HER2-low therapy per the clinical trial design, and HER2 scores of “IHC 1+” and “IHC 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 28.

Table 28. Repeatability and intermediate precision of PATHWAY anti-HER2 (4B5) antibody on breast cancer tissues with HER2-low scoring on BenchMark ULTRA Instrument.

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 Instrument

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 in Breast Carcinoma (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 Table 29.

Table 29. Results of the Reader Precision Study for HER2-low on BenchMark ULTRA Instrument.

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 Table 30.

Table 30. Precision Components for Cases in Reader Precision Study for HER2-low on BenchMark ULTRA Instrument.

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 “IHC 0“ and “IHC 3+” were grouped together as negative cases because they were ineligible for HER2-low therapy per the clinical trial design, and HER2 scores of “IHC 1+” and “IHC 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 below.

Table 31. Within and Between-Reader Precision of the PATHWAY anti-HER2 (4B5) antibody with HER2-low scoring on BenchMark ULTRA Instrument.

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 Instrument

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 Table 32.

Table 32. Results of the Inter-Laboratory Reproducibility Study for HER2-low on BenchMark ULTRA Instrument.

Case	Majority HER2 IHC Score	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)
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)

Case	Majority HER2 IHC Score	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
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 Table 33.

Table 33. Precision Components for Cases in the Inter-Laboratory Reproducibility Study for HER2-low on BenchMark ULTRA Instrument.

Case	Case category	HER2 IHC Score	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 HER2-low on BenchMark ULTRA instrument. For the purposes of study analysis, HER2 scores "IHC 0" and "IHC 3+" were grouped together as negative cases because they were ineligible for HER2-low therapy per the clinical trial design, and HER2 scores of "IHC 1+" and "IHC 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 Table 34.

Table 34. Inter-Laboratory Reproducibility for overall agreement rates for PATHWAY anti-HER2 (4B5) antibody with HER2-low scoring on BenchMark ULTRA Instrument.

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 35.

Table 35. Inter-Laboratory Reproducibility Pairwise Agreement Rates for the PATHWAY anti-HER2 (4B5) antibody with HER2-low scoring on BenchMark ULTRA instrument.

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.

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

Three external laboratories participated in a concordance study between the BenchMark ULTRA PLUS instrument and the BenchMark ULTRA instrument. Tissue slides from 160 breast cancer cases (80 positive and 80 negative, including 16 borderline) were stained with PATHWAY anti-HER2 (4B5) antibody and a negative reagent control internally at Roche Tissue Diagnostics (RTD) on a BenchMark ULTRA instrument using the recommended staining protocol (U PATHWAY HER2 4B5). The ULTRA stained slides were read by one RTD reader and a total of 4 external readers at the 3 laboratories to determine the HER2-low status (negative: HER2 scores of IHC 0 and 3+ or positive: HER2 scores of IHC 1+ and 2+) of these cases. For data analysis, each external ULTRA result was matched with the RTD ULTRA result from 4 scoring categories as shown in Table 36.

Unstained tissue slides from the 160 cases were randomized and equally distributed to the external laboratories for staining on a BenchMark ULTRA PLUS instrument using the recommended staining protocol (U PATHWAY HER2 4B5). One or two readers per site read all the BenchMark ULTRA PLUS slides and determined HER2-low status. All external site readers were blinded to the HER2-low status from the ULTRA-stained slides. ULTRA PLUS results from external site readers were compared to case-matched ULTRA results (RTD-external reader combined result). Results are summarized in Table 36.

Table 36. Agreement of HER2-low status for Cases Stained with PATHWAY anti-HER2 (4B5) antibody on the BenchMark ULTRA PLUS versus BenchMark ULTRA Instrument.

BenchMark ULTRA PLUS	BenchMark ULTRA				
	RTD Reader= Positive, External Reader= Positive	RTD Reader= Positive, External Reader= Negative	RTD Reader= Negative, External Reader= Positive	RTD Reader= Negative, External Reader= Negative	Total
Positive	272	11	12	10	305
Negative	5	8	0	158	171
Total	277	19	12	168	476
Percent Positive % (n/N)	98.2 (272/277)	57.9 (11/19)	100.0 (12/12)	6.0 (10/168)	N/A
	% (n/N)		95% CI		
PPA	95.6 (283/296)		(93.1, 97.9)		
NPA	87.8 (158/180)		(81.0, 95.1)		

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

Note: Two-sided 95% CI calculated using the percentile bootstrap method.

Note: This table is describing concordance of ULTRA PLUS and ULTRA with regard to eligibility to HER2 targeted therapy in the HER2 low population, where HER2 positive is an IHC score of 1+ or 2+ and negative is IHC 0 with membrane staining or null. Note that 3+ is excluded from this table.

Inter-Laboratory Reproducibility Study for HER2-low Breast Cancer on BenchMark ULTRA PLUS Instrument

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 archived 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 assay.

Each set of 5 stained slides per sample over five staining days 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 total). 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 is presented in Table 37. In addition, percent Eligible was calculated with regard to HER2-low therapy.

Table 37. Results of the Inter-Laboratory Reproducibility Study for HER2-low on BenchMark ULTRA PLUS Instrument.

Case	Majority HER2 IHC Bin[b]	N of reads	IHC 0, No staining	IHC 0, faint incomplete ≤10%	IHC 1+, faint incomplete >10%	IHC 2+, weak to moderate complete	IHC 3+, intense complete	Percent Eligible % (n/N)[a]			
								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	97 (29/30)	3 (1/30)	0	0	0	0 (0/10)	0 (0/10)	0 (0/10)	0 (0/30)
3	0	30	93 (28/30)	7 (2/30)	0	0	0	0 (0/10)	0 (0/10)	0 (0/10)	0 (0/30)
4	0	30	100 (30/30)	0	0	0	0	0 (0/10)	0 (0/10)	0 (0/10)	0 (0/30)
5	0	30	100 (30/30)	0	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<1+	30	7 (2/30)	60 (18/30)	33 (10/30)	0	0	20 (2/10)	30 (3/10)	50 (5/10)	33 (10/30)
8	>0<1+	30	17 (5/30)	83 (25/30)	0	0	0	0 (0/10)	0 (0/10)	0 (0/10)	0 (0/30)
9	1+	30	0	0	57 (17/30)	43 (13/30)	0	100 (10/10)	100 (10/10)	100 (10/10)	100 (30/30)
10	1+	30	0	0	77 (23/30)	23 (7/30)	0	100 (10/10)	100 (10/10)	100 (10/10)	100 (30/30)
11	1+	30	0	7 (2/30)	93 (28/30)	0	0	100 (10/10)	80 (8/10)	100 (10/10)	93 (28/30)
12	1+	30	0	23 (7/30)	73 (22/30)	3 (1/30)	0	80 (8/10)	90 (9/10)	60 (6/10)	77 (23/30)
13	1+	30	0	0	70 (21/30)	30 (9/30)	0	100 (10/10)	100 (10/10)	100 (10/10)	100 (30/30)
14	1+	30	0	0	90 (27/30)	10 (3/30)	0	100 (10/10)	100 (10/10)	100 (10/10)	100 (30/30)
15	1+	30	0	0	83 (25/30)	13 (4/30)	3 (1/30)	100 (10/10)	100 (10/10)	90 (9/10)	97 (29/30)
16	1+	30	0	7 (2/30)	80 (24/30)	13 (4/30)	0	100 (10/10)	100 (10/10)	80 (8/10)	93 (28/30)
17	2+	30	0	0	0	100 (30/30)	0	100 (10/10)	100 (10/10)	100 (10/10)	100 (30/30)
18	2+	30	0	0	3 (1/30)	97 (29/30)	0	100 (10/10)	100 (10/10)	100 (10/10)	100 (30/30)
19	2+	30	0	0	40 (12/30)	60 (18/30)	0	100 (10/10)	100 (10/10)	100 (10/10)	100 (30/30)
20	2+	30	3 (1/30)	0	7 (2/30)	90 (27/30)	0	100 (10/10)	100 (10/10)	90 (9/10)	97 (29/30)
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	0	0	3 (1/30)	97 (29/30)	0	100 (10/10)	100 (10/10)	100 (10/10)	100 (30/30)
23	3+	30	0	0	0	0	100 (30/30)	0 (0/10)	0 (0/10)	0 (0/10)	0 (0/30)
24	3+	30	0	0	0	0	100 (30/30)	0 (0/10)	0 (0/10)	0 (0/10)	0 (0/30)
25	3+	30	0	0	0	17 (5/30)	83 (25/30)	0 (0/10)	30 (3/10)	20 (2/10)	17 (5/30)
26	3+	30	0	0	0	0	100 (30/30)	0 (0/10)	0 (0/10)	0 (0/10)	0 (0/30)
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)

[a] Eligible = A HER2 score of 1+ or 2+.

[b] Majority HER2 IHC bin is the most frequent HER2 IHC bin for each case based on the combined study readers' results.

In addition, pairwise comparisons of HER2 (4B5) status were made between sites, readers, and days. For each case, median %TC and range of %TC of 30 results were calculated. As summarized in Table 38, the assay was reproducible across 5 days, 3 sites, and 6 readers.

Table 38. Precision Components for Cases in the Inter-Laboratory Reproducibility Study for HER2-low on BenchMark ULTRA PLUS Instrument.

Case	Case category	HER2 IHC bin[a]	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
2	No staining	0	30	0.0	0 - 1	0.0	0.0	0.0	0.1
3	No staining	0	30	0.0	0 - 1	0.0	0.0	0.0	0.1
4	No staining	0	30	0.0	0 - 0	0.0	0.0	0.0	0

Case	Case category	HER2 IHC bin[a]	N of Reads	Median %TC	Range %TC (Min-Max)	SD			
						Between-reader	Between-day	Between-site	Total
5	No staining	0	30	0.0	0 - 0	0.0	0.0	0.0	0
6	No staining	0	30	0.0	0 - 1	0.0	0.0	0.0	0.1
7	Faint incomplete ≤10%	>0<1+	30	1.0	0 - 8	1.6	0.2	0.0	2.3
8	Faint incomplete ≤10%	>0<1+	30	5.0	0 - 30	5.9	3.3	0.0	8.9
9	Faint incomplete >10%	1+	30	13.5	2 - 70	2.6	11.1	0.0	16.0
10	Faint incomplete >10%	1+	30	15.0	3 - 60	8.5	4.0	0.0	14.4
11	Faint incomplete >10%	1+	30	16.5	11 - 70	10.9	4.4	0.0	14.9
12	Faint incomplete >10%	1+	30	20.0	11 - 60	11.4	6.0	0.0	15.2
13	Faint incomplete >10%	1+	30	22.0	8 - 90	10.0	0.0	0.0	22.0
14	Faint incomplete >10%	1+	30	40.0	20 - 80	8.5	0.0	5.8	16.7
15	Faint incomplete >10%	1+	30	42.0	12 - 95	13.0	0.0	9.6	26.5
16	Faint incomplete >10%	1+	30	50.0	11 - 90	18.0	16.3	0.0	30.5
17	Weak to moderate complete	2+	30	30.0	11 - 64	11.9	0.0	0.0	20.9
18	Weak to moderate complete	2+	30	34.0	0 - 90	27.9	0.0	0.0	37.0
19	Weak to moderate complete	2+	30	50.0	11 - 90	23.3	15.5	0.0	30.9
20	Weak to moderate complete	2+	30	60.0	12 - 95	15.2	0.0	6.4	26.4
21	Weak to moderate complete	2+	30	60.0	30 - 95	17.1	0.7	0.0	22.7
22	Weak to moderate complete	2+	30	70.0	20 - 99	16.5	7.0	0.0	24.8
23	Intense complete	3+	30	85.0	15 - 100	7.4	6.2	0.0	16.4
24	Intense complete	3+	30	90.0	50 - 100	6.3	5.3	7.6	12.5
25	Intense complete	3+	30	90.0	82 - 100	3.2	1.6	2.4	5.5
26	Intense complete	3+	30	95.0	80 - 100	1.1	1.2	4.4	5.9
27	Intense complete	3+	30	99.0	90 - 100	2.1	0.0	0.4	2.7
28	Intense complete	3+	30	99.0	80 - 100	2.3	1.3	1.3	4.2

[a] HER2 IHC bin is based on the majority status of available reads for that case.

Table 39. Inter-laboratory reproducibility for overall agreement rates for PATHWAY anti-HER2 (4B5) antibody 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.

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

Table 40. Inter-laboratory reproducibility pairwise agreement rates for PATHWAY anti-HER2 (4B5) antibody with HER2-low scoring in breast carcinoma on the BenchMark ULTRA PLUS instrument.

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.

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

Repeatability and Precision for HER2-positive Breast Cancer

Repeatability and Intermediate Precision for HER2-positivity on BenchMark XT and BenchMark ULTRA instruments

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 IHC 0, IHC 1+, IHC 2+, and IHC 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 IHC 0, IHC 1+, IHC 2+, and IHC 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 for HER2-positivity on BenchMark XT

Lot-to-Lot precision was determined by automated staining of 5 breast cancer tissues with scores of IHC 0, IHC 1+, IHC 2+, and IHC 3+ HER2 expression with 3 lots of PATHWAY anti-HER2 (4B5) antibody. Stained tissues were scored on a IHC 0 to IHC 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 (IHC 0-1+, IHC 2+, IHC 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 RTD. 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 IHC 0 and IHC 1+, which are both considered to be negative. Two samples (2/40 or 5%) varied between IHC 2+ and IHC 3+, and one sample (1/40) varied between IHC 1+ and IHC 2+. In all of the 40 cases (100%), a minimum of 2 of 3 pathologists agreed.

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

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

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 IHC score (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 IHC score (i.e., 0, 1+, 2+, 3+). 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 41, Table 42 and Table 43 are 3x3 presentations of results for each reader based on clinical score where 2+ and 3+ were separated.

Table 41. 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 A	Site B			
	IHC 3+	IHC 2+	IHC 0, IHC 1+	Total
IHC 3+	12	2	0	14
IHC 2+	0	6	2	8
IHC 0, IHC 1+	0	1	25	26
Total	12	9	27	48
Overall percent agreement (OPA): n/N (%)			43/48 (89.6)	

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

Table 43. 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 B	Site C			
	IHC 3+	IHC 2+	IHC 0, IHC 1+	Total
IHC 3+	12	0	0	12
IHC 2+	0	5	4	9
IHC 0, IHC 1+	0	0	27	27
Total	12	5	31	48
Overall percent agreement (OPA): n/N (%)			44/48 (91.7)	

Instrument Inter day Staining Precision for HER2-positivity on BenchMark ULTRA Instrument

The inter day reproducibility (IDR) portion of the study included 12 cases with an intended distribution of approximately three (3) cases at each clinical IHC 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 IHC score (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 IHC score (i.e., 0, 1+, 2+, 3+) for each case. The results were analyzed by RTD. 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 IHC score (0/1+, 2+, 3+) are shown in Table 44, Table 45, and Table 46.

Table 44. BenchMark ULTRA vs. BenchMark XT Instrument Inter-Platform Agreement Rates 3x3 Analysis – Reader A.

BenchMark ULTRA Instrument	BenchMark XT Instrument			
Reader A	IHC 3+	IHC 2+	IHC 0, IHC 1+	Total
IHC 3+	84	11	1	96
IHC 2+	8	28	9	45
IHC 0, IHC 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 45. BenchMark ULTRA vs. BenchMark XT Instrument Inter-Platform Agreement Rates 3x3 Analysis – Reader B.

BenchMark ULTRA Instrument	BenchMark XT Instrument			
Reader B	IHC 3+	IHC 2+	IHC 0, IHC 1+	Total
IHC 3+	64	2	1	67
IHC 2+	3	56	7	66
IHC 0, IHC 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 46. BenchMark ULTRA vs. BenchMark XT Instrument Inter-Platform Agreement Rates 3x3 Analysis – Reader C.

BenchMark ULTRA Instrument	BenchMark XT Instrument			
Reader C	IHC 3+	IHC 2+	IHC 0, IHC 1+	Total
IHC 3+	64	1	0	65
IHC 2+	2	45	1	48
IHC 0, IHC 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 on BenchMark ULTRA Instrument

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 47 and Table 48.

Table 47. Inter-Pathologist Pairwise Agreement Rates on BenchMark ULTRA Instrument.

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 48. Inter-Pathologist Pairwise Agreement Rates on BenchMark XT Instrument.

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 on BenchMark ULTRA instrument

The Site 1 cohort of 140 FFPE invasive breast carcinoma cases (approximately 35 cases from each HER-2 binning category IHC score (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 IHC score (0/1+, 2+, 3+) are shown in Table 49, Table 50, and Table 51.

Table 49. 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	IHC 3+	IHC 2+	IHC 0, IHC 1+	Total
IHC 3+	43	5	0	48
IHC 2+	3	17	6	26
IHC 0, IHC 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 50. 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	IHC 3+	IHC 2+	IHC 0, IHC 1+	Total
IHC 3+	32	0	0	32
IHC 2+	0	31	6	37
IHC 0, IHC 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 51. 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	IHC 3+	IHC 2+	IHC 0, IHC 1+	Total
IHC 3+	32	0	0	32
IHC 2+	0	23	2	25
IHC 0, IHC 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 on BenchMark ULTRA instrument

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 52 and Table 53.

Table 52. *i*VIEW DAB Detection Kit Inter-Pathologist Reproducibility Agreement Rates on BenchMark ULTRA Instrument.

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 53. *ultra*View Universal DAB Detection Kit Inter-Pathologist Reproducibility Agreement Rates on BenchMark ULTRA Instrument.

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)

Concordance Between BenchMark ULTRA PLUS and BenchMark ULTRA Instruments

Three external laboratories participated in a concordance study between the BenchMark ULTRA PLUS instrument and the BenchMark ULTRA instrument. Tissue slides from 160 breast cancer cases (80 positive and 80 negative, including 16 borderline) were stained with PATHWAY anti-HER2 (4B5) antibody and a negative reagent control internally at RTD on a BenchMark ULTRA instrument using the recommended staining protocol (U PATHWAY HER2 4B5). The ULTRA stained slides were read by one RTD reader and a total of 4 external readers at the 3 laboratories to determine the HER2 status (negative: HER2 scores of IHC 0 and 1+ or positive: HER2 scores of IHC 2+ and 3+) of these cases. For data analysis, each external ULTRA result was matched with the RTD ULTRA result to form 4 scoring categories as shown in Table 54.

Unstained tissue slides from the 160 cases were randomized and equally distributed to the external laboratories for staining on a BenchMark ULTRA PLUS instrument using the recommended staining protocol (U PATHWAY HER2 4B5). One or two readers per site read all the BenchMark ULTRA PLUS slides and determined the HER2 status. All external site readers were blinded to the HER2 status from the ULTRA stained slides. ULTRA PLUS results from external site reader(s) were compared to case-matched ULTRA results (RTD-external reader combined result). The results are summarized in Table 54.

Table 54. Agreement of HER2-positive status for Cases Stained with PATHWAY anti-HER2 (4B5) Assay on the BenchMark ULTRA PLUS versus BenchMark ULTRA 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)

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

Note: Two-sided 95% CI calculated using the percentile bootstrap method.

Note: This table is describing concordance of ULTRA PLUS and ULTRA with regard to eligibility to HER2 targeted therapy in the HER2 positive population, where HER2 positive is an IHC score of 2+ or 3+ and negative is IHC 1+, IHC 0 with membrane staining or null.

Note: When excluding ULTRA/ULTRA PLUS paired observations where at least one observation is HER2 null, the agreement rates (% (95% C.I.)) are as follows: PPA 93.3% (89.3, 96.6), and NPA 90.5% (85.8, 94.9).

Inter-Laboratory Reproducibility Study for HER2-positivity Breast Cancer on BenchMark ULTRA PLUS instrument

An Inter-Laboratory Reproducibility Study of the PATHWAY anti-HER2 (4B5) antibody was conducted to evaluate 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 PATHWAY anti-HER2 (4B5) antibody.

Each set of 5 stained slides per sample over 5 staining days 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 55.

Table 55. Inter-laboratory reproducibility for overall agreement rates for PATHWAY anti-HER2 (4B5) antibody with HER2-positive scoring in breast carcinoma on the BenchMark ULTRA PLUS instrument.

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.

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 and summarized in Table 56.

Table 56. Inter-laboratory reproducibility pairwise agreement rates for PATHWAY anti-HER2 (4B5) antibody with HER2-positive scoring in breast carcinoma on the BenchMark ULTRA PLUS instrument.

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.

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.

Repeatability and Precision - Biliary Tract Cancer

Intermediate Precision for HER2 in BTC on BenchMark ULTRA Instrument

Intermediate Precision was evaluated using biliary tract cancer (BTC, i.e. gallbladder adenocarcinoma, intrahepatic cholangiocarcinoma, and extrahepatic cholangiocarcinoma) samples supplemented with samples from carcinomas of the digestive system (CDS). Twenty-eight CDS samples (10 BTC, 10 gastro-esophageal adenocarcinoma (GEA), and 8 colorectal carcinoma (CRC) samples) spanning the HER2 IHC staining range were included in this study. The study design for evaluation of staining precision on BTC tissues stained with PATHWAY anti-HER2 (4B5) antibody included:

- Three lots of PATHWAY anti-HER2 (4B5) antibody (between antibody kit lot)
- Three lots of *ultra*View Universal DAB IHC Detection Kits (between detection kit lot)
- Across five non-consecutive days (between day)
- Three BenchMark ULTRA instruments (between instrument)
- Across all intermediate precision conditions (within run)
- 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 in Biliary tract Cancer. Each case had 22 results and a majority HER2 score result was assigned based on 22 results. For each case, a median %TC and range of %TC of 22 results was calculated. In addition, the percent Eligible with regard to HER2-positive therapy for BTC was calculated. Results of this analysis are presented in Table 57.

Table 57. Median and Range of %TC for Cases in the Intermediate Precision Study for BTC tissues (supplemented with CDS) on BenchMark ULTRA Instrument.

Case Number	Majority HER2 IHC Score	Median %TC	Range %TC (Min-Max)	Percent Results "Eligible"
1	0	0.0	0.0 - 0.0	0.0% (0/22)
2	0	0.0	0.0 - 0.0	0.0% (0/22)
3	0	0.0	0.0 - 0.0	0.0% (0/22)
4	0	0.0	0.0 - 0.0	0.0% (0/22)
5	0	0.0	0.0 - 0.0	0.0% (0/22)
6	0	0.0	0.0 - 0.0	0.0% (0/22)
7	0	1.0	0.0 - 1.5	0.0% (0/22)
8	0	1.5	0.5 - 7.5	0.0% (0/22)
9	0	3.0	0.5 - 5.5	0.0% (0/22)
10	1+	10.5	10.0 - 17.0	0.0% (0/22)
11	1+	10.5	10.0 - 23.5	0.0% (0/22)
12	1+	12.5	10.0 - 20.0	0.0% (0/22)
13	1+	17.0	1.0 - 30.0	0.0% (0/22)
14	1+	20.5	15.0 - 26.0	0.0% (0/22)
15	2+	15.0	10.0 - 20.0	0.0% (0/22)
16	2+	20.0	15.0 - 25.0	0.0% (0/22)
17	2+	20.0	15.0 - 30.0	0.0% (0/22)
18	2+	20.0	15.0 - 45.5	0.0% (0/22)
19	2+	34.0	25.5 - 57.0	0.0% (0/22)
20	2+	35.0	20.0 - 55.0	0.0% (0/22)
21	2+	37.5	30.0 - 65.0	0.0% (0/22)
22	3+	30.0	15.0 - 50.0	100.0% (22/22)
23	3+	30.0	18.0 - 45.0	100.0% (22/22)
24	3+	52.5	30.0 - 80.0	100.0% (22/22)
25	3+	65.0	60.0 - 70.0	100.0% (22/22)
26	3+	85.0	70.0 - 95.0	100.0% (22/22)
27	3+	90.0	85.0 - 95.0	100.0% (22/22)
28	3+	97.0	93.0 - 100.0	100.0% (22/22)

Variability of %TC values for each case was evaluated and the following precision components were calculated: repeatability (within-run), between-day, between-antibody lot, between-detection kit, between-instrument and total. Results are summarized in Table 58.

Table 58. Precision Components for Cases in Intermediate Precision Study for BTC tissues (supplemented with CDS) on BenchMark ULTRA instrument.

Case Number	Majority HER2 IHC Score	Median %TC	SD					
			Repeatability (within-run)	Between- day	Between- antibody lot	Between-detection kit	Between-instrument	Total
1	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7	0	1.00	0.11	0.36	0.00	0.44	0.00	0.58
8	0	1.50	0.86	2.54	0.00	0.97	0.00	2.85
9	0	3.00	0.11	1.71	0.00	0.00	0.00	1.71
10	1+	10.50	0.15	0.32	0.00	3.68	2.73	4.60
11	1+	10.50	0.54	1.15	0.00	0.00	6.89	7.01
12	1+	12.50	0.32	2.77	0.00	0.00	3.65	4.59
13	1+	17.00	N/A*	N/A*	N/A*	N/A*	N/A*	N/A*
14	1+	20.50	0.30	3.01	1.77	0.00	4.30	5.55
15	2+	15.00	1.95	2.96	0.00	0.00	0.00	3.55
16	2+	20.00	2.61	0.98	0.00	3.20	0.00	4.24
17	2+	20.00	3.37	0.00	4.63	0.75	1.63	6.00
18	2+	20.00	2.38	1.85	15.29	0.00	0.00	15.59
19	2+	34.00	2.36	5.95	10.36	0.00	13.32	18.05
20	2+	35.00	3.20	4.99	0.00	3.10	9.42	11.55
21	2+	37.50	2.82	5.04	10.2	0.00	14.92	18.97
22	3+	30.00	3.37	9.48	4.81	4.81	0.00	12.15
23	3+	30.00	3.53	7.63	8.18	0.00	0.00	11.73
24	3+	52.50	6.91	8.54	0.00	21.63	0.00	24.26
25	3+	65.00	2.13	2.42	2.54	0.00	0.46	4.13
26	3+	85.00	4.13	6.82	3.10	0.00	3.10	9.09
27	3+	90.00	2.82	1.01	0.00	0.00	3.71	4.77
28	3+	97.00	1.09	1.99	0.00	0.00	1.95	2.99

* The ANOVA analyses of %TC were performed only if all replicates were in the same IHC bin (0/1+, 2+, or 3+). N/A denotes that not all replicates were in the same IHC bin for this case.

In addition, a qualitative analysis of different precision components was performed. For the purposes of study analysis, HER2 IHC scores of “0”, “1+”, and "2+" were considered negative and a HER2 score of “3+” was considered positive. Precision was determined with positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA) across all observations. A summary of the results can be found in Table 59.

Table 59. Repeatability and intermediate precision of PATHWAY anti-HER2 (4B5) antibody on BTC tissues (supplemented with CDS) on BenchMark ULTRA Instrument.

Repeatability/Precision	Agreement			
	Type	n/N	%	95% CI
Between-Antibody Lots	PPA	42/42	100.0	(91.6, 100.0)
	NPA	126/126	100.0	(97.0, 100.0)
	OPA	168/168	100.0	(97.8, 100.0)
Between-Detection Kits	PPA	42/42	100.0	(91.6, 100.0)
	NPA	126/126	100.0	(97.0, 100.0)
	OPA	168/168	100.0	(97.8, 100.0)
Between- Instrument (BenchMark ULTRA)	PPA	42/42	100.0	(91.6, 100.0)
	NPA	126/126	100.0	(97.0, 100.0)
	OPA	168/168	100.0	(97.8, 100.0)
Between-Day	PPA	70/70	100.0	(94.8, 100.0)
	NPA	210/210	100.0	(98.2, 100.0)
	OPA	280/280	100.0	(98.6, 100.0)
Within-Run	PPA	77/77	100.0	(95.2, 100.0)
	NPA	231/231	100.0	(98.4, 100.0)
	OPA	308/308	100.0	(98.8, 100.0)

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

Reader Precision for HER2 in BTC on BenchMark ULTRA Instrument

In the Reader Precision study, Between-Reader and Within-Reader components of precision were evaluated. The study included 37 BTC samples supplemented with 28 GEA samples and 25 CRC samples spanning the HER2 IHC staining range. Seventy-five (75) resection samples and 15 biopsy samples were included. Samples were blinded and randomized prior to evaluation for HER2 IHC score using the Criteria for Intensity and Pattern of Cell Membrane Staining with PATHWAY anti-HER2 (4B5) Antibody in Biliary tract Cancer. The study included three readers (Pathologists). Each reader scored all specimens twice, with a minimum of two weeks between reads. Each case had six reads (two reads by each of three readers). Results from the 75 resection samples included in the Reader Precision study are presented in Table 60.

Table 60. Results of the Reader Precision study in BTC resection samples (supplemented with CDS) on BenchMark ULTRA Instrument.

Case Category	HER2 IHC	N of Cases	N of Reads	Results by HER2 IHC Score			
				0	1+	2+	3+
No reactivity < 10%	0	16	96	96	0	0	0
Faint/barely perceptible < 10% / ≥ 10%	0/1+	8	48	21	27	0	0
Faint/barely perceptible ≥ 10% / weak to moderate complete, basolateral or lateral	1+/2+	1	6	0	3	3	0
Weak to moderate complete, basolateral or lateral	2+	25	150	0	0	150	0
Weak to moderate complete, basolateral or lateral / Strong complete, basolateral or lateral	2+/3+	2	12	0	0	6	6
Variable	0/1+/2+	1	6	4	1	1	0
Strong complete, basolateral or lateral	3+	22	132	0	0	0	132

The variability of %TC for cases included in the Reader Precision study was evaluated and the following precision components were calculated: within-reader, between-reader, and total. Results from the 75 resection samples are summarized in Table 61.

Table 61. Precision Components for Cases in Reader Precision Study for BTC resection samples (supplemented with CDS) on BenchMark ULTRA Instrument.

Case Category	HER2 IHC	N of cases	N of reads	Range of median %TC	SD			Percent Results "Eligible"
					Within-Reader	Between-Reader	Total	
No reactivity < 10%	0	16	96	0.0 - 1.5	1.1	0.2	1.2	0.0% (0/96)
Faint/barely perceptible < 10% / ≥ 10%	0/1+	8	48	2.0 - 12.5	4.3	3.9	5.8	0.0% (0/48)
Faint/barely perceptible ≥ 10% / weak to moderate complete, basolateral or lateral	1+/2+	1	6	17.5 - 17.5	N/A	N/A	N/A	0.0% (0/6)
Weak to moderate complete, basolateral or lateral	2+	25	150	11.0 - 60.0	11.8	13.0	17.6	0.0% (0/150)
Weak to moderate complete, basolateral or lateral / Strong complete, basolateral or lateral	2+/3+	2	12	15.0 - 47.5	N/A	N/A	N/A	50.0% (6/12)
Variable	0/1+/2+	1	6	5.0 - 5.0	N/A	N/A	N/A	0.0% (0/6)
Strong complete, basolateral or lateral	3+	22	132	20.0 - 98.0	12.1	4.4	12.9	100.0% (132/132)

In addition, a qualitative analysis of within-reader and between-reader precision was determined with average positive agreement (APA), average negative agreement (ANA), and overall percent agreement across all observations. For the purposes of study analysis, HER2 IHC scores of “0”, “1+”, and "2+" were considered negative, and a HER2 score of “3+” was considered positive. The agreement for between-reader and within-reader components for all samples (resections and biopsies) are summarized in Table 62.

Table 62. Within- and Between-Reader Precision of the PATHWAY anti-HER2 (4B5) antibody in BTC tissues (supplemented with CDS) on BenchMark ULTRA Instrument

Precision	Agreement			
	Type	n/N	%	95% CI
Within-Reader	APA	178/180	98.9	(97.2, 100.0)
	ANA	358/360	99.4	(98.6, 100.0)
	OPA	268/270	99.3	(98.1, 100.0)
Between Reader	APA	180/180	100	(97.9, 100.0)
	ANA	360/360	100	(98.9, 100.0)
	OPA	270/270	100	(98.6, 100.0)

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

Inter-Laboratory Reproducibility Study for HER2 (4B5) with BTC on BenchMark ULTRA Instrument

An Inter-Laboratory Reproducibility study of the PATHWAY anti-HER2 (4B5) antibody was conducted to evaluate reproducibility of the assay to determine HER2-status of BTC specimens stained on the BenchMark ULTRA instrument. The study included 28 de-identified FFPE BTC tissue specimens stained on 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 over 5 staining days was randomized and evaluated by a total of 6 readers (2 readers / site) for a HER2 IHC score. Each case had 10 results per site (30 results total). For each case, the percent positive with regard to HER2-positive therapy in BTC was calculated. Results of this analysis for each case are presented in Table 63.

Table 63. Results of the Inter-Laboratory Reproducibility Study in BTC on BenchMark ULTRA Instrument.

Case Number	Majority HER2 IHC Score	N of Reads	HER2 IHC Score				Percent Positive Result			
			0	1+	2+	3+	Site A	Site B	Site C	Overall
1	0	30	21 (70.0)	8 (26.7)	1 (3.3)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
2	0	30	30 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
3	0	30	30 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
4	0	30	30 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
5	0	28	28 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.0 (0/10)	0.0 (0/8)	0.0 (0/10)	0.0 (0/28)
6	0	30	14 (46.7)	10 (33.3)	6 (20.0)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
7	0	30	26 (86.7)	4 (13.3)	0 (0.0)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
8	0	30	29 (96.7)	1 (3.3)	0 (0.0)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
9	0	30	20 (66.7)	7 (23.3)	3 (10.0)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
10	0	30	30 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
11	0	30	20 (66.7)	10 (33.3)	0 (0.0)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
12	0	30	30 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
13	1+	30	12 (40.0)	17 (56.7)	1 (3.3)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
14	1+	30	7 (23.3)	19 (63.3)	4 (13.3)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
15	2+	30	0 (0.0)	0 (0.0)	30 (100.0)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
16	2+	30	4 (13.3)	8 (26.7)	18 (60.0)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
17	2+	30	0 (0.0)	0 (0.0)	30 (100.0)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
18	2+	30	0 (0.0)	0 (0.0)	30 (100.0)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
19	2+	30	0 (0.0)	2 (6.7)	28 (93.3)	0 (0.0)	0.0 (0/10)	0.0 (0/10)	0.0 (0/10)	0.0 (0/30)
20	2+	30	1 (3.3)	1 (3.3)	25 (83.3)	3 (10.0)	0.0 (0/10)	0.0 (0/10)	30.0 (3/10)	10.0 (3/30)
21	2+	30	0 (0.0)	0 (0.0)	21 (70.0)	9 (30.0)	20.0 (2/10)	30.0 (3/10)	40.0 (4/10)	30.0 (9/30)
22	3+	30	0 (0.0)	0 (0.0)	0 (0.0)	30 (100.0)	100.0 (10/10)	100.0 (10/10)	100.0 (10/10)	100.0 (30/30)
23	3+	30	0 (0.0)	0 (0.0)	0 (0.0)	30 (100.0)	100.0 (10/10)	100.0 (10/10)	100.0 (10/10)	100.0 (30/30)
24	3+	30	0 (0.0)	0 (0.0)	6 (20.0)	24 (80.0)	80.0 (8/10)	60.0 (6/10)	100.0 (10/10)	80.0 (24/30)
25	3+	30	0 (0.0)	0 (0.0)	5 (16.7)	25 (83.3)	60.0 (6/10)	100.0 (10/10)	90.0 (9/10)	83.3 (25/30)
26	3+	30	0 (0.0)	0 (0.0)	5 (16.7)	25 (83.3)	50.0 (5/10)	100.0 (10/10)	100.0 (10/10)	83.3 (25/30)
27	3+	30	0 (0.0)	0 (0.0)	0 (0.0)	30 (100.0)	100.0 (10/10)	100.0 (10/10)	100.0 (10/10)	100.0 (30/30)
28	3+	30	0 (0.0)	0 (0.0)	0 (0.0)	30 (100.0)	100.0 (10/10)	100.0 (10/10)	100.0 (10/10)	100.0 (30/30)

In addition, a qualitative analysis of different precision components was performed. For the purposes of study analysis, HER2 IHC scores of "0", "1+", and "2+" were considered negative and a HER2 IHC score of "3+" was considered positive.

The data were analyzed for positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA) across all evaluable observations, and a summary is presented in Table 64.

Table 64. Inter-Laboratory Reproducibility for overall agreement rates for PATHWAY anti-HER2 (4B5) antibody in BTC on BenchMark ULTRA Instrument

Inter-Laboratory Reproducibility	Agreement			
	Type	n/N	%	95% CI
Overall	PPA	194/210	92.4	(85.2, 97.6)
	NPA	616/628	98.1	(95.2, 100.0)
	OPA	810/838	96.7	(94.0, 99.0)
Within-Site	PPA	194/210	92.4	(85.2, 97.6)
	NPA	616/628	98.1	(95.2, 100.0)
	OPA	810/838	96.7	(94.0, 99.0)
Within-Reader	PPA	200/210	95.2	(89.5, 99.5)
	NPA	622/628	99.0	(97.3, 100.0)
	OPA	822/838	98.1	(96.2, 99.8)

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

In addition, pairwise comparisons were made Between-Site, Between-Reader, and Between-Day for HER2 clinical status. These data were analyzed for average positive agreement (APA), average negative agreement (ANA), and overall percent agreement (OPA) and are presented in Table 65.

Table 65. Inter-Laboratory Reproducibility Pairwise Agreement Rates for PATHWAY anti-HER2 (4B5) antibody in BTC on BenchMark ULTRA Instrument

Inter-Laboratory Reproducibility	Agreement			
	Type	n/N	%	95% CI
Between-Site	APA	3636/4120	88.3	(79.7, 96.3)
	ANA	12116/12600	96.2	(93.3, 98.8)
	OPA	7876/8360	94.2	(90.0, 98.2)
Between-Reader	APA	182/206	88.3	(78.6, 96.5)
	ANA	608/632	96.2	(92.7, 98.9)
	OPA	395/419	94.3	(89.0, 98.3)
Between-Day	APA	772/824	93.7	(87.5, 99.0)
	ANA	2468/2520	97.9	(95.7, 99.7)
	OPA	1620/1672	96.9	(93.7, 99.5)

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

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 HER-2 (CB11) Mouse Monoclonal 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 for 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.[17,18,19] A total of 322 cases were evaluated. The slides stained with PATHWAY HER-2 (CB11) Mouse Monoclonal Antibody were processed and stained according to the manufacturer's instructions specified in the PATHWAY HER-2 (CB11) Mouse Monoclonal Antibody package insert (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 in Table 66, Table 67, Table 68, Table 69, Table 70, and Table 71.

Inter-pathologist Reproducibility of Comparison Studies Specimens

Table 66. Cohort 1: Consensus IHC Scores of Three Pathologists.

4B5 Score	CB11 Score			Total
	IHC 3+	IHC 2+	IHC 0, IHC 1+	
IHC 3+	29	24	5	58
IHC 2+	2	13	17	32
IHC 0, IHC 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 IHC (2+ and 3+) and negative IHC (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 67. Cohort 2: Consensus IHC Scores of Three Pathologists.

4B5 Score	CB11 Score			Total
	IHC 3+	IHC 2+	IHC 0, IHC 1+	
IHC 3+	72	1	0	73
IHC 2+	1	12	5	18
IHC 0, IHC 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 IHC (2+ and 3+) and negative IHC (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 68. Cohort 1: Consensus CB11 IHC Scores of Three Pathologists Compared to FISH.

CB11 Score	FISH Result		Total
	Positive	Negative	
IHC 3+	32	0	32
IHC 2+	32	5	37
IHC 0, IHC 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 69. Cohort 1: Consensus 4B5 IHC Scores of Three Pathologists Compared to FISH

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

Cohort 1: Performance characteristics for 4B5 and FISH, 2 x 2 Presentation (where scores of IHC 2+ and IHC 3+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 70. Cohort 2: Consensus CB11 IHC Scores of Three Pathologists Compared to FISH

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

Cohort 2: Performance characteristics for CB11 and FISH, 2 x 2 Presentation (where scores of IHC 2+ and IHC 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 71. Cohort 2: Consensus 4B5 IHC Scores of Three Pathologists: Compared to FISH

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

Cohort 2: Performance characteristics for 4B5 and FISH, 2 x 2 Presentation (where scores of IHC 2+ and IHC 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 72. Cohort 1: 4B5 Scoring for the Three Pathologists

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

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

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

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

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

Table 73. Cohort 1: CB11 Scoring for the Three Pathologists

HER2 Score	CB11 Score		
	Investigator 1	Investigator 2	Investigator 3
IHC 3+	72	75	73
IHC 2+	22	22	18
IHC 0, IHC 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 74. Cohort 2: 4B5 Scoring for the Three Pathologists

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

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

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

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

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

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

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

Table 75. Cohort 2: CB11 Scoring for the Three Pathologists

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

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

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

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

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

Table 76. 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 PERFORMANCE IN BREAST CANCER

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 77 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 77. 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 78 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 78. Efficacy results from KATHERINE.

Parameter	KADCYLA N= 573	Trastuzumab N= 559
Primary Endpoint	Invasive Disease Free Survival (IDFS)*	
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** %	89.0	75.7

* Data from first interim analysis

** 3-year event-free rate derived from Kaplan-Meier estimates

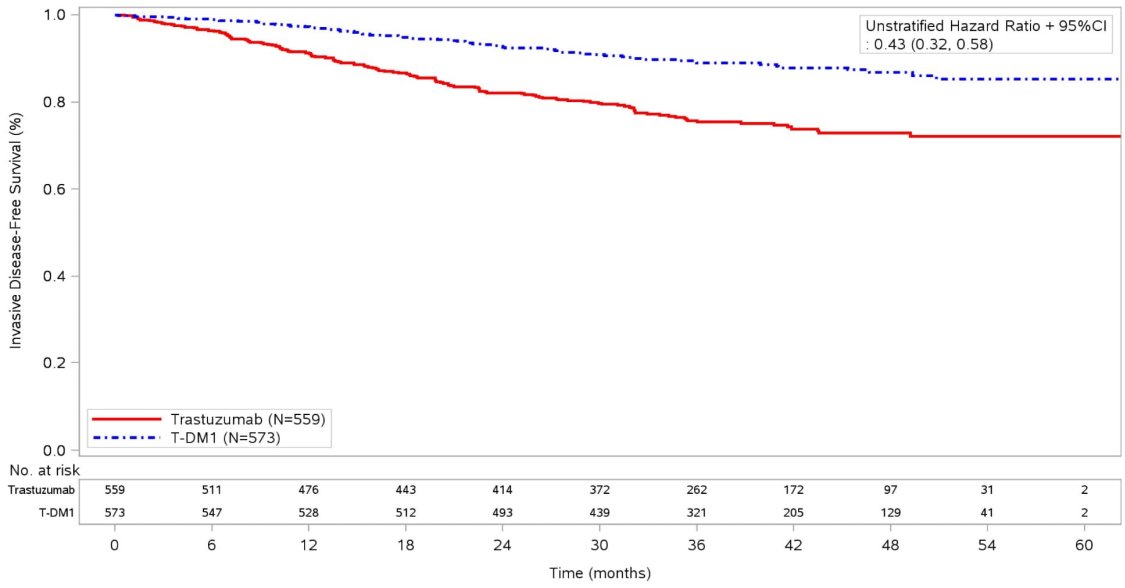

Figure 2. Kaplan-Meier curve of invasive disease free survival in KATHERINE.

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 (ER) and/or progesterone receptor (PgR)).

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 79).

Table 79. Efficacy Results in DESTINY-Breast04.

Efficacy Parameter	HR+ Cohort		Overall Population (HR+ and HR- Cohorts)	
	ENHERTU® (N = 331)	Chemotherapy (N = 163)	ENHERTU® (N = 373)	Chemotherapy (N = 184)
Progression-Free Survival per BICR				
Median*, 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** (95% CI)	0.51 (0.40, 0.64)		0.50 (0.40, 0.63)	
P-value***	< 0.0001		< 0.0001	
Overall Survival				
Median* (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** (95% CI)	0.64 (0.48, 0.86)		0.64 (0.49, 0.84)	
P-value***	0.0028		0.0010	

CI = confidence interval, PFS = progression-free survival, OS = overall survival, BICR = blinded independent central review

* 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.

** 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).

*** Two-sided P-value from stratified log-rank test.

Clinical Outcome Study – DESTINY-Breast06

DESTINY-Breast06 is a phase III randomized, multicenter, open-label, active controlled trial evaluating the safety and efficacy of fam-trastuzumab deruxtecan-nxki (ENHERTU®) in hormone receptor positive breast cancer (BC) patients whose disease had progressed on endocrine therapy in the metastatic setting with HER2-low or HER2-ultralow expression levels, centrally confirmed using the PATHWAY anti-HER2 (4B5) antibody. A tumor with a HER2 IHC score IHC 0 with membrane staining (described as IHC >0<1+ in this study) was considered to have a HER2-ultralow status. A tumor with a HER2 IHC score of IHC 1+ was considered to have a HER2-low status. A tumor was also considered HER2-low if the HER2 IHC score was IHC 2+ and reflex testing with ISH indicated the absence of HER2 gene amplification (ISH-). Enrolled patients were randomized 1:1 to receive either fam-trastuzumab deruxtecan-nxki (ENHERTU®) or physician's choice chemotherapy treatment. The HER2 status was one of 3 stratification factors used for patient randomization.

The primary efficacy outcome measure was PFS in patients with HER2-low breast cancer assessed by BICR based on RECIST v1.1. Key secondary efficacy outcome measures were PFS assessed by BICR based on RECIST v1.1 in the overall population (HER2-low and HER2-ultralow), OS in HER2-low patients, and OS in the overall population.

Patients randomized to T-DXd had a statistically significant and clinically meaningful improvement in PFS as assessed by BICR compared with patients randomized to chemotherapy across the study populations which included HER2-low (IHC1+ or IHC2+/ISH-) population and the overall population (HER2 IHC 0 with membrane staining, IHC1+ and IHC2+/ISH-).

Overall survival (OS) data were immature (39%) at the time of analysis.

Efficacy results are summarized in Table 80.

Table 80. PFS per BICR and ORR in DESTINY-Breast06.

Efficacy Parameter	HER2-Low		Overall Population (HER2-Low and HER2-Ultralow)	
	ENHERTU® (N=359)	Chemotherapy (N=354)	ENHERTU® (N=436	Chemotherapy (N=430)
Progression Free Survival (PFS) per BICR				
Number of events (%)	225 (62.7)	232 (65.5)	269 (61.7)	271 (63.0)
Median PFS, months (95% CI)	13.2 (11.4, 15.2)	8.1 (7.0, 9.0)	13.2 (12.0, 15.2)	8.1 (7.0, 9.0)
Hazard Ratio (95% CI)	0.62 (0.52, 0.75)*		0.64 (0.54, 0.76)**	
P-value	< 0.0001*		< 0.0001**	
Confirmed Objective Response Rate (ORR) per BICR***				
N	326	324	393	389
n (%)	202 (62.0)	114 (35.2)	246 (62.6)	134 (34.4)
95% CI	56.5, 67.3	30.0, 40.7	57.6, 67.4	29.7, 39.4
Complete Response n (%)	9 (2.8)	0	10 (2.5)	0
Partial Response n (%)	193 (59.2)	114 (35.2)	236 (60.1)	134 (34.4)
Duration of Response (DOR) per BICR***				
Median, months (95% CI)	14.1 (11.9, 15.9)	8.6 (6.7, 11.3)	14.3 (12.5, 15.9)	8.6 (6.9, 11.5)

CI = confidence interval, PFS = progression-free survival, ORR = objective response rate, BICR = blinded independent central review

* Based on stratified analysis with stratification factors prior CDK4/6 inhibitor use (yes vs no) and HER2 IHC status of tumor samples (IHC 1+ vs IHC 2+/ISH-).

** Based on unstratified analysis.

*** Analysis was performed based on the patients with measurable disease assessed by BICR at baseline.

Clinical Outcome Study – DESTINY-Breast09

The efficacy of ENHERTU® in combination with PERJETA® (pertuzumab) was evaluated in DESTINY-Breast09 (NCT04784715), a Phase 3, randomized, three-arm, multicenter, global study that enrolled 1157 adult patients with HER2-positive (IHC 3+ or ISH-amplified) advanced, or metastatic breast cancer, as determined using PATHWAY anti-HER2/neu (4B5) and VENTANA HER2 Dual ISH DNA Probe Cocktail. A subset (n=34) of patients was screened and randomized using on-market PATHWAY anti-HER2/neu (4B5) and VENTANA HER2 Dual ISH DNA Probe Cocktail which is identical to the corresponding investigational versions of the assays with respect to reagent formulation, manufacturing process, staining procedure parameters, interpretation of stained tissue and tissue indication, and therefore are functionally identical. A tumor with a HER2 IHC score of IHC 3+ or ISH-amplified (regardless of IHC status) was considered to indicate a HER2 positive status.

The study included adult patients with unresectable or metastatic HER2-positive breast cancer who had not received prior chemotherapy or HER2-targeted therapy for advanced or metastatic breast cancer.

Patients were randomized 1:1:1 to receive either ENHERTU® 5.4 mg/kg plus pertuzumab (N=383), or THP (taxane [docetaxel or paclitaxel], trastuzumab, and pertuzumab) (N=387), or an investigational therapy (N=387) by intravenous infusion every 3 weeks. The primary efficacy outcome was progression-free survival (PFS) as assessed by blinded independent central review (BICR) based on (RECIST) v1.1. An additional efficacy outcome measure was overall survival (OS).

The median age was 54 years (range: 20-88); 82% were <65 years; 100% were female; 50% of the patients were Asian, 37.2% were White, 2.1% were American Indian or Alaska Native, 14% were of Hispanic/Latino ethnicity, and 2.6% were black or African American; the remainder were of another race or race unknown. The percentage of patients who had de novo disease was 52.2% and recurrent disease was 47.8%. The percentage of patients who were HR positive was 53.2%; HR negative was 46.8%; and 30.9% of patients had a PIK3CA mutation.

At the time of the PFS analysis, 15.1% of patients in the combination arm and 17.6% of patients in the THP arm had died. Overall survival (OS) was immature. Table 81 summarizes the efficacy results for the full analysis set and full analysis set IHC3+ subset, for ENHERTU® in combination with pertuzumab compared to THP. Although DESTINY-Breast09 was not designed to compare between the investigational and on-market selected subpopulations, very similar PFS results were observed in the investigational device subset.

Table 81. Efficacy Results in DESTINY-Breast09

Efficacy Parameter	Full Analysis Set (IHC3+ or ISH-Amplified)		Full Analysis Set IHC 3+ Subset	
	ENHERTU® + pertuzumab	THP	ENHERTU® + pertuzumab	THP
Progression Free Survival (PFS) per BICR				
N	383	387	318	315
Number of events (%)	118 (30.8)	172 (44.4)	91 (28.6)	136 (43.2)
Median, months (95% CI)	40.7 (36.5, NE)	26.9 (21.8, NE)	40.7 (36.5, NE)	27.6 (22.4, NE)
Hazard Ratio (95% CI)	0.56 (0.44, 0.71)		0.54 (0.41, 0.70)	
P-value	< 0.0001[a]		Not Calculated	

BICR = blinded independent central review, CI = confidence interval, NE = not estimable, PFS = progression-free survival, THP = taxane (docetaxel or paclitaxel), trastuzumab, and pertuzumab

[a] The p-value for Full Analysis Set was calculated using a stratified log-rank test.

CLINICAL PERFORMANCE IN BILIARY TRACT CANCER

Clinical outcome study – HERIZON-BTC-01

The efficacy of zanidatamab-hrii (ZIIHERA®) and the clinical performance of PATHWAY anti-HER2/neu (4B5) Rabbit Monoclonal Primary Antibody were evaluated in 62 patients with HER2-positive (IHC 3+ by central assessment) biliary tract cancer (BTC, i.e. gallbladder adenocarcinoma, intrahepatic cholangiocarcinoma, or extrahepatic cholangiocarcinoma) in Cohort 1 of HERIZON-BTC-01 (NCT04466891), an open label, multicenter, single-arm, trial in patients with unresectable or metastatic disease. Patients were required to have HER2-amplified BTC, to have received at least one prior gemcitabine-containing systemic chemotherapy regimen in the advanced disease setting and to have adequate cardiac function (defined as LVEF ≥ 50%).

Tumor samples from patients undergoing screening were tested with PATHWAY anti-HER2/neu (4B5) Rabbit Monoclonal Primary Antibody to assess HER2 protein expression by IHC.

Patients received zanidatamab-hrii (ZIIHERA®) 20 mg/kg intravenously every 2 weeks. Zanidatamab-hrii (ZIIHERA®) was administered until disease progression or unacceptable toxicity. The major efficacy outcome measures were objective response rate (ORR) and duration of response (DOR) as determined by an independent central review (ICR) according to Response Evaluation Criteria in Solid Tumors (RECIST) v1.1. Efficacy results (data cutoff date - DCO: 28 July 2023) are summarized in Table 82.

Table 82. Efficacy Results in ZWI-ZW25-203.

Efficacy Parameter*	ZIHERA® (N=62)
Objective Response Rate (95% CI)	52% (39,65)
Complete response, n (%)	2 (3.2)
Partial response, n (%)	30 (48)
Duration of Response (DOR)**	N=32
Median**, months (95% CI)	14.9 (7.4, NE)
DOR ≥ 6 months (95% CI)*** DOR ≥12 months (95% CI)***	19 (59) 14 (44)

NE = not estimable

* Assessed by independent central review

** Based on Kaplan-Meier estimate

*** Based on observed duration of response

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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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.

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
N	Updates to the Intended Use, Scoring Algorithm (Table 3) and Clinical Performance sections

INTELLECTUAL PROPERTY

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