VENTANA HER2 Dual ISH DNA Probe Cocktail

REF 800-6043

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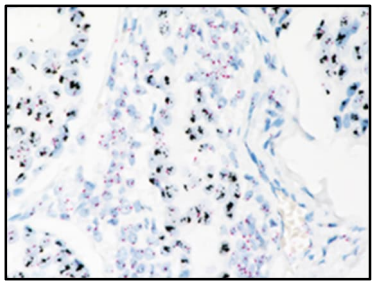


Figure 1. Amplified HER2 status with VENTANA HER2 Dual ISH DNA Probe Cocktail assay, Breast Carcinoma.

INTENDED USE

The VENTANA HER2 Dual ISH DNA Probe Cocktail is intended to determine *HER2* gene status by enumeration of the ratio of the *HER2* gene to Chromosome 17 by light microscopy. The HER2 and Chromosome 17 probes are detected using two-color chromogenic in situ hybridization (ISH) in formalin-fixed, paraffin-embedded human breast and gastric carcinoma tissue specimens, including the gastroesophageal junction, following staining on BenchMark IHC/ISH instruments.

The VENTANA HER2 Dual ISH DNA Probe Cocktail is indicated as an aid in the assessment of patients for whom Herceptin (trastuzumab) is being considered. This product 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

Human epidermal growth factor receptor 2 (HER2) 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] Approximately 15 to 30 percent of breast carcinomas demonstrate overexpression of the HER2 protein, amplification of the HER2 gene (*ERBB2*), or both.[3,4] Knowledge of *HER2* gene and/or protein status in invasive breast carcinoma enables clinicians to make more informed decisions to improve the overall management of care for these patients.[5] HER2 status is an established predictive factor for response to HER2 targeted therapy in breast cancer patients.[5,6,7]

Trastuzumab (Herceptin) is a humanized monoclonal antibody against the extracellular domain of HER2 and has been shown to benefit patients with HER2 positive breast cancer.[8-13] Demonstration of *HER2* gene amplification and/or protein overexpression is essential for selecting patients for trastuzumab therapy.[5,14]

Similarly, *HER2* gene amplification or protein overexpression occurs in gastric and gastroesophageal junction adenocarcinoma (collectively referred to as gastroesophageal adenocarcinoma or GEA).[15,16,17] A wide range of HER2 overexpression frequency has been reported across published studies. However, one of the largest screening datasets which included 3,803 patients with GEA reported that 22 percent of patients tested positive for HER2 protein expression or gene amplification.[18] The majority of studies suggest that in the absence of HER2 directed therapy, HER2 overexpression is a negative prognostic factor.[19]

The HER2 targeted therapy trastuzumab is a mainstay in the management of invasive breast carcinoma and has therapeutic value in the management of gastric/GEA cancer patients overexpressing the receptor.[15,17] Demonstration of HER2 gene amplification and/or protein overexpression is essential for selecting patients for trastuzumab therapy.[15,19] Clinical studies have shown that breast or gastric/GEA cancer patients with high HER2 protein overexpression and/or gene amplification benefit most from trastuzumab.[3,15]

PRINCIPLE OF THE PROCEDURE

The VENTANA HER2 Dual ISH DNA Probe Cocktail contains HER2 probes (labeled with the hapten dinitrophenyl or DNP) and Chromosome 17 probes (labeled with the hapten digoxigenin or DIG) formulated in a formamide-based buffer. The probes are designed to detect amplification of the *HER2* gene in invasive breast carcinoma and GEA. The HER2 DNA Probe is a mixture of oligo probes that specifically targets the *HER2* gene (also known as ERBB2 and NEU), which is located on human Chromosome 17 (17q12). The Chromosome 17 probe is a mixture of oligo probes that target sequences within the centromeric region and serves as a reference for aneusomy. Copy numbers of both probes are enumerated in tumor nuclei and results are reported as a ratio of *HER2*/Chromosome 17 to determine *HER2* amplification status (*HER2*/Chromosome 17 ratio ≥ 2.0 is amplified, while a ratio < 2.0 is non-amplified). The VENTANA HER2 Dual ISH DNA Probe Cocktail is optimally formulated for use with VENTANA Silver ISH DNP Detection Kit, VENTANA Red ISH DIG Detection Kit, and accessory reagents on a BenchMark IHC/ISH instrument.

The detection kit contains a primary antibody and an enzyme-labeled secondary antibody conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP) which is used as the chromogenic enzyme. During the Dual in situ hybridization (Dual ISH) staining process, DNP and DIG labeled probes are co-hybridized to their respective specific target DNA sequences within the cell nuclei. Detection of the DNP-labeled HER2 probe occurs first, using the VENTANA Silver ISH (SISH) DNP Detection Kit, which contains the following dispensers: mouse anti-DNP primary antibody labeled with hydroxyquinoxaline (HQ), mouse anti-HQ secondary antibody conjugated to horseradish peroxidase (HRP), Chromogen A (Silver A), Chromogen B (Silver B) and Chromogen C (Silver C). Following incubation with the HQ-labeled mouse anti-DNP primary antibody and then mouse anti-HQ HRP secondary antibody conjugate, the SISH reaction occurs. Briefly described, this reaction is driven by the sequential addition of Chromogens A (silver acetate), B (hydroquinone) and C (H_2O_2). Here, the silver ions (Ag^+) are reduced by hydroquinone to metallic silver atoms (Ag^0). This reaction is fueled by the substrate for HRP, hydrogen peroxide (Chromogen C). The silver precipitate is deposited in the nuclei and a single copy of the *HER2* gene is visualized as a black dot. Figure 2 illustrates the SISH reaction.

Following SISH detection for *HER2*, the DIG-labeled Chromosome 17 probe is detected with the VENTANA Red ISH DIG Detection Kit. This kit includes the following dispensers: mouse anti-DIG primary antibody labeled with nitropyrazole (NP), mouse anti-NP secondary antibody conjugated to Alkaline Phosphatase (AP), pH Enhancer, Naphthol, and Fast Red. Following development of the SISH signal, the slide is incubated with the NP-labeled mouse anti-DIG primary antibody, which binds to the DIG hapten on the Chromosome 17 probe. The anti-hapten primary antibody is detected with the mouse anti-NP conjugated to AP enzyme. The slide is incubated with the pH Enhancer solution, which provides the proper salt components and concentrations and buffered pH for optimal AP enzyme performance. Next, naphthol phosphate is applied, which serves as the substrate for the AP enzyme (AP dephosphorylates naphthol). Fast Red, added to the slide next, combines with the dephosphorylated naphthol to form a red precipitate, which is readily visualized by light microscopy. Figure 3 illustrates the Red ISH reaction. The specimen is then counterstained with Hematoxylin II for interpretation by light microscopy.

The staining protocol consists of numerous steps in which reagents are incubated for pre-determined times at specific temperatures. At the end of each incubation step, the BenchMark IHC/ISH instrument washes the sections to remove unbound material and applies a liquid coverslip which minimizes the evaporation of the aqueous reagents from the slide. Results are interpreted using a light microscope using 20x, 40x, and/or 60x objectives.

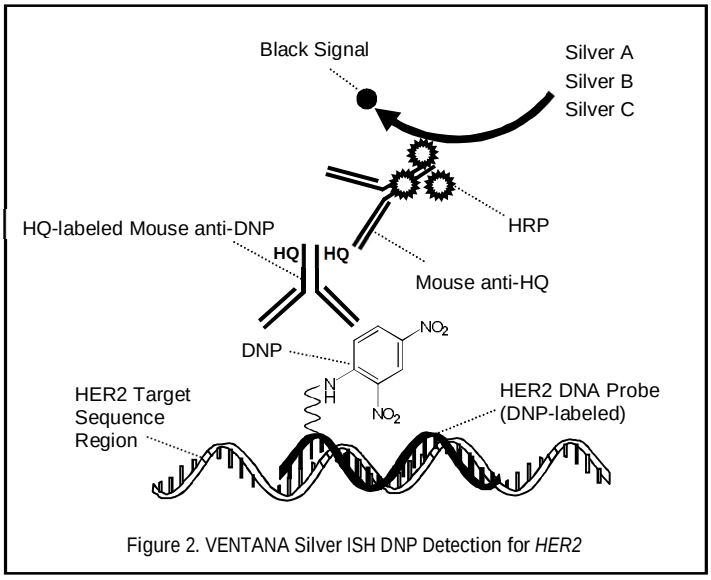


Figure 2. VENTANA Silver ISH DNP Detection for *HER2*

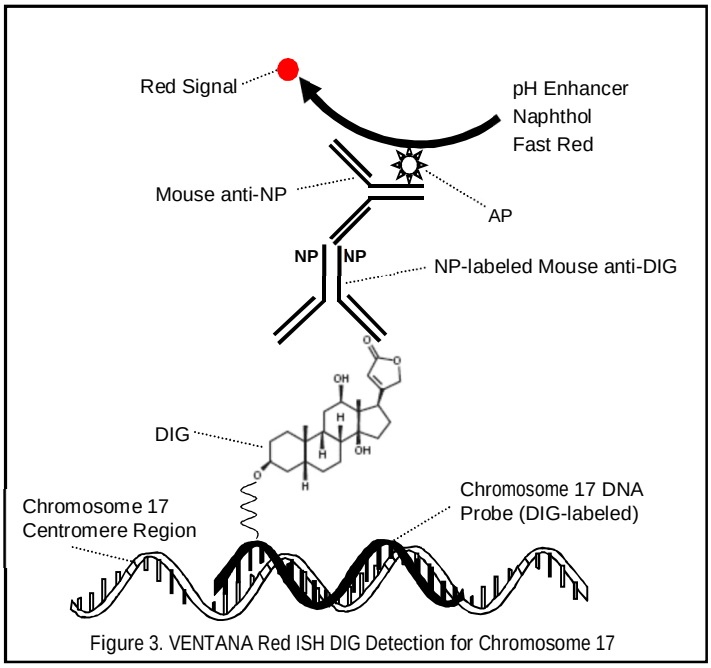


Figure 3. VENTANA Red ISH DIG Detection for Chromosome 17

MATERIAL PROVIDED

The VENTANA HER2 Dual ISH DNA Probe Cocktail dispenser contains sufficient reagent for 30 tests.

One 6 mL dispenser of VENTANA HER2 Dual ISH DNA Probe Cocktail contains approximately 14 µg/mL of the HER2 probes labeled with dinitrophenyl (DNP) and 0.24 µg/mL of the Chromosome 17 probes labeled with digoxigenin (DIG) formulated in a formamide-based hybridization buffer. Both probes are used to determine *HER2* gene status (i.e., ratio of *HER2*/Chromosome 17).

Refer to the appropriate VENTANA detection kit method sheets for detailed descriptions of: Principles of the Procedure, Materials 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, are not provided.

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

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

1. VENTANA Silver ISH DNP Detection Kit (Cat. No. 760-516 / 08318883001)
2. VENTANA Red ISH DIG Detection Kit (Cat. No. 760-512 / 08318832001)
3. HybReady Solution (Cat. No. 780-4409 / 05917557001)
4. ISH Protease 3 (Cat. No. 780-4149 / 05273331001)
5. Hematoxylin II (Cat. No. 790-2208 / 05277965001)
6. Bluing Reagent (Cat. No. 760-2037 / 05266769001)
7. Reaction Buffer Concentrate (10X) (Cat. No. 950-300 / 05353955001)
8. SSC (10X) (Cat. No. 950-110 / 05353947001)
9. EZ Prep Concentrate (10X) (Cat. No. 950-102 / 05279771001)
10. *ultra*View Silver Wash II (Pre-dilute) (Cat. No. 780-003 / 05446724001)
11. Cell Conditioning Solution (CC1) (Cat. No. 950-124 / 05279801001)
12. Cell Conditioning Solution (CC2) (Cat. No. 950-123 / 05279798001)
13. LCS (Predilute) (Cat. No. 650-010 / 05264839001)
14. ULTRA LCS (Pre-dilute) (Cat. No. 650-210 / 05424534001)
15. ULTRA Cell Conditioning Solution (ULTRA CC1) (Cat. No. 950-224 / 05424569001)
16. ULTRA Cell Conditioning Solution (ULTRA CC2) (Cat. No. 950-223 / 05424542001)
17. BenchMark IHC/ISH instrument
18. Microscope slides, positively charged (Superfrost Plus or equivalent)
19. Permanent mounting medium*
20. Cover slip sufficient to cover tissue
21. Automated coverslipper
22. HER2 Dual ISH 3-in-1 Xenograft Slides (Cat. No. 783-4422 / 05640300001) can be used for troubleshooting activities, as needed.

* See Table 30 for compatible mounting media with this assay.

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 probe, replace the dispenser cap after every use and immediately place the dispenser in the refrigerator in an upright position.

Every probe 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, formalin-fixed, paraffin-embedded (FFPE) tissues are suitable for use with this probe when used with a BenchMark IHC/ISH instrument. The recommended tissue fixative is 10% neutral buffered formalin (NBF) for 6 to 72 hours.[20] Aside from the VENTANA assays, studies have found that the majority of inconclusive *HER2* gene results by FISH relate to pre-analytic factors including under- and over-fixation,[21] as well as delayed fixation.[22] Strict implementation of fixation procedures (e.g., a dedicated processor to ensure a minimum of 6 hours fixation) resulted in a 68.5% reduction in inconclusive cases from 10.8% failures to 3.4%. Specimens fixed < 6 hours in formalin can result in signal loss and nuclear over-digestion, as observed by pale/weak hematoxylin staining. Only fixation in 10% NBF is recommended as some fixatives produce variable staining with ISH-based assays (including Bouin's and Alcohol Formalin-Acetic Acid (AFA)).[21]

Slides should be stained immediately, as quality of nucleic acid targets in cut tissue sections may diminish over time. Internal studies have shown that breast and gastric cut slides stored at 2-8 °C can be stable for 12 months. Positively charged slides may be susceptible to environmental stresses resulting in inappropriate staining of any ISH assay (for example, lack of staining or counterstain on the tissue). Ask your Roche representative for a copy of "Impact of environmental stress on various histology slide types" to better understand how to use these types of slides.

Each section should be cut to the appropriate thickness (4 µm) for the assay used and placed on positively charged microscope slides (Superfrost Plus or equivalent). Slides should be drained or dried to remove excess water between slide and tissue.

Sections thicker than 4 µm may require stronger protease treatment than the recommended condition and may exhibit more nuclear bubbling than thinner sections due to excess paraffin in the tissue. Nuclear bubbling appears as large or small bubbles or vacuoles in the nuclei. Often when nuclear bubbling occurs there is a spectrum of effects on the SISH and Red ISH signals characterized by 1) nuclei with nuclear bubbles in which the SISH and Red ISH signals are generally still centrally located in the nucleus and 2) nuclei with nuclear bubbles that push the SISH and Red ISH signals to the periphery. Often in both cases, if the SISH and Red ISH signals are clearly discernable, are not otherwise distorted, and are still enumerable, the case can be scored. However, occasionally severe nuclear bubbling may distort the SISH and Red ISH signals or make them indiscernible such that accurate enumeration is not possible. This occurs more often when SISH and Red ISH signals are pushed to the nuclear periphery. When this occurs one can often find nuclei elsewhere in the sample that are enumerable and the case can be scored. If nuclear bubbling is severe, to the degree that one cannot find sufficient nuclei in which SISH and Red ISH signals can be confidently enumerated, the case should not be scored. Nuclear bubbling also may occur in the context of underfixation (1-3 hours with formalin), which is a less discrete nuclear bubbling. This may be remedied at 3 hours fixation with changed cell conditioning/protease treatment, but at 1 hour is probably beyond remedy.

The VENTANA HER2 Dual ISH DNA Probe Cocktail assay has been developed with additional pre-treatment options that may aid in optimizing the assay in different laboratories and for subsequent troubleshooting of particular tissues / slides exhibiting sub-optimal staining. It is recommended that each laboratory perform initial runs on representative control samples that have been prepared under the identical conditions as the clinical samples to be tested. This will aid in optimizing the specific staining conditions for individual laboratories that may vary in their exact specimen preparation procedures. Variable results may occur with different pre-analytical factors than recommended. Specimens that are pre-analytically prepared using conditions that are not recommended may never stain appropriately with the assay.

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. Warning, Product Contains Formamide. Formamide is toxic by inhalation and moderately toxic by ingestion. It is an irritant to skin, eyes, and mucous membranes and is absorbed through the skin. It may cause harm to the unborn child. Take precautions when handling reagents. Use disposable gloves and wear suitable protective clothing when handling suspected carcinogens or toxic materials.
5. Materials of human or animal origin should be handled as potentially biohazardous and disposed of with proper precautions. In the event of exposure, the health directives of the responsible authorities should be followed.[23,24]
6. Avoid contact of reagents with eyes and mucous membranes. If reagents come in contact with sensitive areas, wash with copious amounts of water. Avoid inhalation of reagents.
7. Ensure that the waste container is empty prior to starting a run on the instrument. If this precaution is not taken, the waste container may overflow and the user risks a slip and fall.
8. Avoid microbial contamination of reagents as this may produce incorrect results.
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 to determine the 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.

This product contains components classified as follows in accordance with the Regulation (EC) No. 1272/2008:

Table 1. Hazard information.

Hazard	Code	Statement
DANGER 	H351	Suspected of causing cancer.
	H360D	May damage the unborn child.
	H373	May cause damage to organs through prolonged or repeated exposure.
	P201	Obtain special instructions before use.
	P202	Do not handle until all safety precautions have been read and understood.
	P260	Do not breathe mist or vapours.
	P280	Wear protective gloves/ protective clothing/ eye protection/ face protection/ hearing protection.
	P308 + P313	If exposed or concerned: Get medical advice/ attention.
	P501	Dispose of contents/ container to an approved waste disposal plant.

This product contains CAS# 75-12-7: formamide

STAINING PROCEDURE

VENTANA probes have been developed for use on a BenchMark IHC/ISH instrument in combination with VENTANA detection kits and accessories. The staining procedures for the BenchMark IHC/ISH instrument with the VENTANA Silver ISH DNP Detection Kit and the VENTANA Red ISH DIG Detection Kit are listed in Table 2. The recommended staining protocols are listed in Table 3.

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

For more details on the proper use of this device, refer to the inline dispenser method sheet associated with P/N 800-6043.

Table 2. Use the following staining procedures to perform VENTANA HER2 Dual ISH DNA Probe Cocktail assay on BenchMark IHC/ISH instruments.

Instrument Platform	Staining Procedure
BenchMark GX	GX VENTANA HER2 DISH DNA PRB CKT
BenchMark XT	XT VENTANA HER2 DISH DNA PRB CKT
BenchMark ULTRA or BenchMark ULTRA PLUS	U VENTANA HER2 DISH DNA PRB CKT

Table 3. Recommended staining conditions for VENTANA HER2 Dual ISH DNA Probe Cocktail assay on BenchMark IHC/ISH instruments.

Staining Condition	Breast	Gastric
Baking	Not selected	Not selected
Cell Conditioning 1	16 mins	16 mins
Cell Conditioning 2	24 mins	16 mins
ISH Protease 3	20 mins	16 mins
Stringency Wash Temperature	76°C for BenchMark GX/XT instruments	76°C for BenchMark GX/XT instruments

Staining Condition	Breast	Gastric
	74°C for BenchMark ULTRA or ULTRA PLUS instruments	74°C for BenchMark ULTRA or ULTRA PLUS instruments

Due to variation in tissue fixation and processing, as well as general lab instrument and environmental conditions, it may be necessary to increase or decrease the cell conditioning or protease pretreatment based on individual specimens.

Starting a Run on BenchMark IHC/ISH instruments

1. Apply slide bar code label that corresponds to the probe protocol to be performed.
2. Load the VENTANA HER2 Dual ISH DNA Probe Cocktail, reagents from VENTANA Red ISH DIG and VENTANA Silver ISH DNP Detection Kits, and required accessory reagents into the reagent tray(s) or carousel. Place reagent tray(s) or carousel on the instrument.
3. Check bulk fluids and waste.
4. The reaction buffer bulk bottles must be full.
5. The waste container must be empty prior to the start of the run.
6. Load slides onto the instrument.
7. Start the staining run.
8. At the completion of the run, remove slides from the instrument. The stained slides will have residual buffer and liquid coverslip solution on them. Proceed with rinsing and dehydration (see below).

Dehydration Procedure

Note: The Fast Red chromogen is soluble in alcohol and acetone. Stained slides exposed to alcohol and/or acetone can result in a loss of specific signal.

1. To remove liquid coverslip solution, wash slides in 2 sequential solutions of a mild dishwashing detergent (do not use detergent designed for automatic dishwashers).
2. Rinse slides well with distilled water, about 1 minute. Shake off excess water.
3. Place slides in an oven (45-60°C) to dry or air dry at ambient temperature. In an oven, drying times range from 10 minutes to one hour (drying stained slides for a longer period of time does not appear to impact staining results). Ensure slides are completely dry before coverslipping, as residual water on the slides can interfere with the coverslipping procedure and cause bubbles to form.
4. Transfer slides into xylene bath for approximately 30 seconds.
5. Place mounting media on slide.
6. Place coverslip on slide. Note that some mounting media are not compatible with the assay and should not be used (See Limitations and Troubleshooting sections).

QUALITY CONTROL PROCEDURES

Positive Control Specimen

Normal *HER2* and Chromosome 17 signals (1 to 2 copies per cell) act as internal positive controls and must be visible in the sample using 20x, 40x, and/or 60x objectives. However, not all cells will exhibit single gene copy due to biological heterogeneity. Specific nuclear staining may be located in various cells including: stromal fibroblasts, endothelial cells, lymphocytes, and non-neoplastic epithelial cells. If the positive controls fail to demonstrate positive staining, this may indicate a reagent or instrument problem. Since every specimen has an internal positive control (i.e., appropriate ISH staining in normal cells), this acts as the true “positive control”.

A laboratory-specific positive specimen control may be used with every staining procedure performed. Control specimens can be specimens prepared in a manner identical to patient specimens. Such controls are useful to monitor all steps of the procedure, from specimen preparation through staining. Use of a specimen prepared differently from the test specimens will provide a control for the reagents, instrument and procedures but not for fixation and specimen processing. Results with the test specimens should be analyzed on the same run. Such controls should not replace the proper evaluation of the internal controls in each patient specimen.

Xenograft Specimen

Xenograft slides may be useful for a preliminary validation of the method used for staining slides with VENTANA HER2 Dual ISH DNA Probe Cocktail assay. They also are recommended as aids for troubleshooting, when used in runs containing clinical samples. For more information, see the appropriate xenograft slide method sheet.

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 method sheet. Identify and correct the problem, then repeat the patient samples.

Assay Verification

Prior to initial use of a probe or staining system in a diagnostic procedure, the specificity of the probe should be verified by testing it on a series of tissues with known ISH performance characteristics (refer to the probe method sheet and to the Quality Control recommendations of the College of American Pathologists Laboratory Accreditation Program, Anatomic Pathology Checklist,[25] or the CLSI Approved Guideline[26] or both documents). These quality control procedures should be repeated for each new lot of reagents, or whenever there is a change in assay parameters.

STAINING INTERPRETATION / EXPECTED RESULTS

The cellular staining pattern for VENTANA HER2 Dual ISH DNA Probe Cocktail assay is nuclear.

A qualified pathologist experienced in the microscopic interpretation of anatomic pathology specimens, ISH procedures and the recognition of single and amplified *HER2* and Chromosome 17 (Chr17) copies (which require microscopic examination using 20x, 40x, and/or 60x objectives) must evaluate controls before interpreting results.

Note: Use of 100x objective is not recommended. All of the tissue slides read during design verification and validation testing were done using 20x, 40x, and/or 60x objectives.

The VENTANA HER2 Dual ISH DNA Probe Cocktail must be used along with the *Interpretation Guide VENTANA HER2 Dual ISH DNA Probe Cocktail* [P/N 1018386] for slide evaluation.

The following sections describe how to interpret and score slides. Table 4 illustrates how to count discrete signals.

Definitions

1. *HER2* Gene Status. *HER2* Gene status is a function of the ratio of the number of copies of the *HER2* gene to the number of copies of Chr17, per cell, in an invasive breast carcinoma or GEA case. *HER2* gene status is classified using the following guidelines:
   a. *HER2*/Chr17 ratio ≥ 2.0 is amplified
   b. *HER2*/Chr17 ratio < 2.0 is non-amplified
2. Slide Adequacy. A VENTANA HER2 Dual ISH DNA Probe Cocktail slide must satisfy three criteria to be deemed adequate for enumeration; if the slide does not meet these criteria, then it cannot be enumerated and the result is unsatisfactory.
   a. Internal Positive Control. Normal *HER2* and Chr17signals (1 to 2 copies per cell) act as internal positive controls and must be visible in the sample. This nuclear staining may be located in various non-neoplastic cells including: stromal fibroblasts, endothelial cells, lymphocytes, and non-neoplastic epithelial cells.
   b. Neoplastic cells. Using 20x, 40x, and/or 60x objectives, the invasive aspect of the tumor must exhibit an enumerable field of SISH and Red ISH signals.
   c. Background. Any background staining resulting from either SISH or Red ISH detection systems will need to be evaluated to determine if it interferes with enumeration of the specific SISH or Red ISH signals. SISH background typically appears as SISH “dust” that is distinguishable from the specific signal. Red background may appear as red haze or rarely nonspecific signals that are fainter in intensity compared to the specific signal.
3. Target Areas for Signal Enumeration. An acceptable target area within the invasive carcinoma exhibits an enumerable field of SISH and Red ISH signals. Signal enumeration should not be performed in areas that contain weak SISH or Red ISH signal, compressed or overlapping nuclei, or necrosis. If one target area is deemed inadequate for enumeration, it often is possible to find other target areas on the same slide that are adequate. This can be determined by the presence of normal cells exhibiting appropriate SISH and Red ISH staining in or adjacent to the target area.

Additional Observations for *HER2* and Chromosome 17

Other observations may be noted as comments on the pathologist’s report.

1. Heterogeneity: In some cases, the tissue may contain areas of carcinoma that are genetically heterogeneous for *HER2* copy number (*i.e.*, there may be a mixture of unamplified and amplified nuclei or a mixture of nuclei containing various copies of

HER2). This may be observed among carcinoma cells within the target area itself, or between two different target areas.

2. Aneusomy is any condition in which an organism has additional or fewer specific chromosomes than normal, i.e., the number of a particular chromosome (in this case, Chromosome 17) is not diploid. In polysomy, there may be three or more copies of the chromosome rather than the expected two copies. In monosomy, the tumor cells may exhibit only one copy of Chromosome 17. Apparent "amplification," clusters, or polysomy of Chromosome 17 (with or without *HER2* SISH clusters) have been reported.[27] In cases with clusters of *HER2* and Chromosome 17, care must be taken not to consider them with a ratio of ~1.0. The reader should refer to immunohistochemistry (IHC) results for HER2 protein overexpression analyses in these cases, as the majority tend to be 3+.
3. Monoallelic Deletion: The deletion of the *HER2* gene from Chromosome 17 in the tumor cells results in a *HER2*/Chr17 ratio < 1.0.

Signal Visualization

SISH and Red ISH signals are visualized as:

1. Single Copy. A discrete black dot (SISH) is counted as a single copy of HER2. Discrete single dots visualized in the internal, control (non-neoplastic) nuclei represent the size of a single copy in invasive carcinoma cells for the SISH (black) signal. For Red ISH signals, each discrete signal is counted as one copy. It should be noted that the Red ISH signal from the Chr17 may appear larger than the SISH signals, and sometimes elongated in shape. Pink haze may occur and should not be mistaken for signal. Red signals that are very light in color compared to the signal in internal positive control nuclei and overall pattern of staining should not be enumerated, as they may be non-specific. Specific red signals have discrete edges, as shown in Table 4.
2. Multiple Copies. Discrete single SISH signals visualized in the internal positive control nuclei represent the size of a single copy *HER2* in invasive carcinoma cells. The size of the single SISH signals is used as a reference to determine the relative number of amplified copies in the cancer nuclei. For Red ISH signals, each discrete signal is counted as one copy.
3. Clusters. Presence of multiple overlapping signals in the nuclei that cannot be enumerated. A cluster is defined as numerous overlapping SISH signals in the nuclei that cannot be individually discerned. Clusters of *HER2* can only be estimated by the reader. For example, a large cluster of multiple SISH signals could be estimated as 12 copies, while smaller clusters may be estimated as 6 copies. The estimation is made by using the single SISH copies present in the internal positive control cells as a reference. The presence of *HER2* clusters is noted on the score sheet.
4. Overlapping nuclei, nuclei with only one color present, and specimens with non-specific staining should not be enumerated. Any nuclei with overlapping Red ISH and SISH signals that cannot be discerned should be visualized at higher magnifications to discern the two signals or should not be counted. Nuclei that appear bubbled should not be counted.

Enumeration of the SISH and Red ISH signals to determine *HER2* gene status

Examine the H&E stained slide to locate areas containing invasive breast or gastroesophageal carcinoma. Examine the HER2 Dual ISH stained slide corresponding to the H&E, and identify an invasive breast or gastroesophageal carcinoma target area. Before enumerating *HER2* and Chromosome 17 signals to determine *HER2* gene status, it is critical to determine whether the invasive target area (the lesional tissue) is adequately stained and satisfies the criteria described for slide adequacy (see the Definitions section above, 2. Slide Adequacy).

The scoring algorithm developed for the assay maximizes precision and efficiency in counting. Twenty nuclei, each containing red (Red ISH) and black (SISH) signals, should be enumerated.

Cell Selection Criteria

Count only nuclei with diameters that are representative of the average population of invasive carcinoma nuclei in the target area. Do not count signals in nuclei that are:

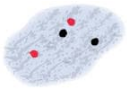
1. Much larger in diameter than the average size of carcinoma nuclei
2. Much smaller in diameter than the average size of carcinoma nuclei

Count only nuclei that are representative of the population of invasive carcinoma nuclei with the highest average number of signals (both SISH and Red ISH).

In target areas that are genetically heterogeneous for HER2 copy number, count only nuclei that are representative of the population of invasive carcinoma nuclei with the highest average number of signals (both SISH and Red ISH). Note that heterogeneity is present on the score sheet.

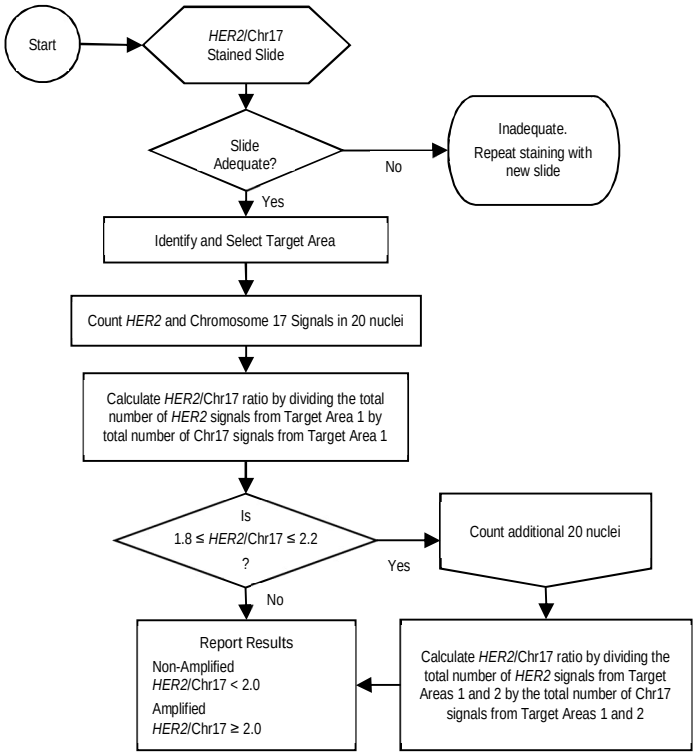
Table 4. Signal Visualization.

	Do not count if nuclei overlap.
	Do not count if no signal is present.
	Do not count if only signal of one color is present.
	Do not count if signals are outside the nuclei.
	Count as 1 black (*HER2*) and 1 red (Chr17) signal.
	Count as 2 black (*HER2*) and 2 red (Chr17) signals.
	Count as 1 black (*HER2*) and 2 red (Chr17) signals. The black signal is a "doublet". Count two adjacent signals of same color only if the distance between the signals is equal to or greater than the diameter of a single signal.
	Small SISH clusters can only be estimated by using the size of a single signal as reference. Use stromal cells to estimate signal size (smaller cell). For instance, this cluster could be estimated as 6 SISH signals - adding the other 2 single signals yields a total count of 8. Count as 2 red signals. Note on scoring sheet that clusters are present for *HER2*.
	Estimate the large cluster. Here, the cluster can be estimated as 12 black signals - adding the other 4 single signals yields a total count of 16. Count red signals as 2 copies of Chr17. Note on scoring sheet that clusters are present for *HER2*.
	A red signal close to a black signal should be counted as one red signal and one black signal. This may require enumeration at 60x objective to discern. Therefore, count as 4 black (*HER2*) and 2 red (Chr17) signals. If overlapping signals cannot be distinguished, do not count that nucleus.
	Cluster of black dots obscuring red signal(s). Higher magnification (60x) may be utilized in attempts to confirm presence or absence of red signal(s); otherwise do not count: always count nuclei with clear red signals. Note the presence of SISH clusters on the score sheet. Nuclei with visible and higher numbers of red signal should be scored in nuclei with SISH clusters.

	If background SISH “dust” occurs in the nuclei, only count if specific SISH signals are clearly distinguishable from background.
	Pink haze may be observed and should not be mistaken for signal. Small, faint Red ISH signals may be seen and could represent nonspecific binding of the Chr17 probe to other chromosomes. The image shows 2 discrete red (Chr17) signals and 2 black (*HER2*) signals.

HER2 Gene Status: Scoring Algorithm for the VENTANA HER2 Dual ISH DNA Probe Cocktail

Twenty nuclei (each containing red (Chr17) and black (*HER2*) signals) should be enumerated. The final results for the HER2 status are reported based on the ratio formed by dividing the sum of *HER2* signals for all 20 nuclei divided by the sum of Chromosome 17 signals for all 20 nuclei. The amplification status is defined as Amplified if the *HER2*/Chr17 ratio ≥ 2.0 and as Non-Amplified if the *HER2*/Chr17 ratio < 2.0. If the *HER2*/Chr17 ratio falls between 1.8 to 2.2, an additional 20 nuclei should be enumerated. A new ratio should then be formed on the basis of all 40 nuclei, and the amplification status reported as already described.



Controls

Normal cells within, or adjacent to, the target area serve as internal controls of the staining. At least 50% of the normal cell nuclei should contain at least one SISH signal and at least 50% should contain at least one Red ISH signal (the SISH and Red ISH signals do not have to be in the same cells) for the target area to be deemed adequate. Failure to detect adequate signal in normal cells on any slide on the run indicates that the particular slide is inadequate for enumeration. Using positive control samples or xenograft slides will aid in troubleshooting potential instrument and/or reagent problems.

LIMITATIONS

General Limitations

1. ISH is a multiple step methodology that requires specialized training in the selection of the appropriate reagents, specimen preparation, processing, preparation of the ISH slide, and interpretation of the 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, reagent trapping, false negative, or false positive results. Inconsistent results may be a consequence of variations in fixation and embedding methods, or inherent irregularities within the tissue.
3. Excessive or incomplete counterstaining may compromise proper interpretation of results.
4. The clinical interpretation of staining must be evaluated within the context of clinical history, morphology and other histopathological criteria. It is the responsibility of a qualified pathologist to be familiar with the reagents and methods used to produce the stained preparation. Staining must be performed in a certified, licensed laboratory under the supervision of a pathologist who is responsible for the review of the stained slides and ensuring the adequacy of controls.
5. VENTANA reagents are provided at optimal dilution for use when the provided instructions are followed. Any deviation from recommended test procedures may invalidate expected results. Users who deviate from recommended test procedures must accept responsibility for interpretation of patient results.
6. Due to variations in specimen processing it may be necessary to either increase or decrease the ISH protease treatment time. Additionally, increasing or decreasing the cell conditioning will affect staining results. Such changes must be validated by the user. Users who deviate from recommended test procedures are responsible for interpretation of patient results under these circumstances.
7. Reagents may demonstrate unexpected reactions in previously untested tissues. The possibility of unexpected reactions even in tested tissue groups cannot be completely eliminated because of biological variability of tissues. Contact your local support representative with documented unexpected reactions.

SPECIFIC LIMITATIONS

1. Not all fixatives are compatible with the assay. The recommended fixative is 10% NBF for 6 to 72 hours.
2. The VENTANA HER2 Dual ISH DNA Probe Cocktail assay was developed to stain tissue sections that are cut at ~4 µm in thickness.[20] Sections thicker than 4 µm may experience tissue loss.
3. All assays might not be registered on every instrument. Please contact your local support representative for more information.
4. Oxidation, fading, and/or disappearance of the SISH signal may be due to certain brands of mounting media. See Table 30 for compatibility of mounting media.
5. To prevent the Red ISH signal from dissolving, stained slides must not be submerged in alcohol or acetone baths for dehydration. Air drying or drying in an oven is recommended. The stained slides must be completely dry before coverslipping.
6. As with any test, a negative result means that the specific target was not detected, not that the specific target was absent in the cells or tissue assayed.
7. This probe has been optimized for use with VENTANA reagents on BenchMark IHC/ISH instruments. Users who deviate from recommended test procedures are responsible for interpretation of patient results under these circumstances.

PERFORMANCE CHARACTERISTICS

The performance of the VENTANA HER2 Dual ISH DNA Probe Cocktail was evaluated through analytical and clinical studies. All staining was performed using the VENTANA HER2 Dual ISH DNA Probe Cocktail protocol as noted in Table 3 on BenchMark IHC/ISH instruments unless otherwise specified.

Table 5 and Table 6 summarize the performance data across these studies: Concordance Studies, Repeatability and Precision, Within Reader and Between Reader Precision, Lot to Lot Precision, Instrument Inter-Laboratory Precision, Analytical Sensitivity and Specificity, Assay Characterization, and Stability. A subset of these studies are described in greater detail in the following sections.

Table 5. Summary of performance results for breast across analytical and clinical studies.

Pass	Fail	Total	Failure Modes			
			Weak/no HER2/Chr17 (internal control or target cells)	Background Failures	No tissue	Other
2893	127	3020	113 (3.74%)	5 (0.17%)	6 (0.20%)	3 (0.10%)

Table 6. Summary of performance results for gastric across analytical and clinical studies.

Pass	Fail	Total	Failure Modes			
			Weak/no HER2/Chr17 (internal control or target cells)	Background Failures	No tissue	Other
1340	17	1357	17 (1.25%)	0 (0%)	0 (0%)	0 (0%)

CLINICAL PERFORMANCE

Concordance Study with PathVysion Assay: VENTANA HER2 Dual ISH DNA Probe Cocktail on BenchMark ULTRA Instrument vs. Abbott/Vysis PathVysion HER-2 DNA Probe Kit

To evaluate the concordance of the VENTANA HER2 Dual ISH DNA Probe Cocktail assay to the comparator device, the Abbott/Vysis PathVysion HER-2 FISH Kit, in determination of *HER2* gene status in invasive breast carcinoma, a multi-site concordance study was performed. Three central laboratories participated for the VENTANA HER2 Dual ISH DNA Probe Cocktail assay testing. Six hundred thirty-six cases of human invasive breast carcinoma were provided from three clinical enrollment sites for potential inclusion in the study based on HER2 protein expression obtained previously with IHC. The study sponsor supplemented 133 cases. The central laboratories conducting the VENTANA HER2 Dual ISH DNA Probe Cocktail assay and the PathVysion HER-2 FISH assay were blinded to IHC status and original case identifier to prevent bias in evaluation of the specimens. One central laboratory performed IHC staining on all samples using PATHWAY anti-HER-2/neu (4B5) Rabbit Monoclonal Primary Antibody (PATHWAY anti-HER2 (4B5) antibody) for the additional analyses. The FISH and VENTANA HER2 Dual ISH DNA Probe Cocktail assay staining results were enumerated by counting at least 20 nuclei in each specimen. The results were reported as: *HER2*/Chr 17 ratio ≥ 2.0 as amplified; *HER2*/Chr 17 < 2.0 as non-amplified. Of the 678 cases that were stained by both the FISH and VENTANA HER2 Dual ISH DNA Probe Cocktail assays, 605 specimens were enumerable by both assays and therefore included in the analysis of agreement rates.

Primary Results

The primary analysis compared positive and negative percent agreement rates to assess concordance between the VENTANA HER2 Dual ISH DNA Probe Cocktail and PathVysion HER-2 FISH assays in breast carcinoma. Data for amplified and non-amplified clinical assessments for each assay, pooling data across all sites, are presented in a 2x2 table below along with positive percent and negative percent agreement rates where PathVysion HER-2 FISH is the reference assay. Acceptance criteria for demonstrating equivalent performance of these two assay methods when using the BenchMark ULTRA instrument required the two-sided 95% score confidence interval lower bounds be 85% or higher when pooling data from all three sites. These acceptance criteria were met (Table 7). Additionally, positive and negative agreement rates by site were all greater than 85% (Table 8).

Table 7. Agreement between VENTANA HER2 Dual ISH DNA Probe Cocktail and Abbott/Vysis PathVysion HER-2 DNA Probe Kit in a cohort of human breast carcinoma specimens.

	PathVysion HER-2 FISH Result		
VENTANA HER2 Dual ISH DNA Probe Cocktail Result	Amplified	Non-Amplified	Total
Amplified	270	12	282
Non-Amplified	32	291	323
Total	302	303	605

	n/N	% (95% Score CI)
Positive Percent Agreement	270/302	89.4 (85.4, 92.4)
Negative Percent Agreement	291/303	96.0 (93.2, 97.7)

Table 8. Summary of negative, positive, and overall agreement rates for VENTANA HER2 Dual ISH DNA Probe Cocktail and Abbott/Vysis PathVysion HER-2 DNA Probe Kit on human breast carcinoma specimens, presented by site.

VENTANA HER2 Dual ISH DNA Probe Cocktail vs PathVysion HER-2 FISH	Positive Percent Agreement	Negative Percent Agreement	Overall Percent Agreement
Site A: n/N (%) (95% CI)	92/100 (92.0%) (85.0, 95.9)	92/93 (98.9%) (94.2, 99.8)	184/193 (95.3%) (91.4, 97.5)
Site B: n/N (%) (95% CI)	93/103 (90.3%) (83.0, 94.6)	108/119 (90.8%) (84.2, 94.8)	201/222 (90.5%) (86.0, 93.7)
Site C: n/N (%) (95% CI)	85/99 (85.9%) (77.7, 91.4)	91/91 (100.0%) (95.9, 100.0)	176/190 (92.6%) (88.0, 95.6)

These data indicate excellent agreement between the VENTANA HER2 Dual ISH DNA Probe Cocktail assay and PathVysion HER-2 FISH Kit in determining *HER2* gene status in human breast carcinoma specimens.

Secondary Results

Overall percent agreement between VENTANA HER2 Dual ISH DNA Probe Cocktail and PathVysion HER-2 FISH Kit and its two-sided 95% score CI, pooling data from all clinical sites, was 92.7% (90.4, 94.5).

Secondary Results: IHC vs. ISH for HER2 status

The concordance study comparing VENTANA HER2 Dual ISH DNA Probe Cocktail and PathVysion FISH was designed to also evaluate cases based on their IHC scores for HER2 protein levels (see PATHWAY anti-HER2 (4B5) antibody method sheet [P/N 14427EN], for IHC scoring). This enabled a secondary analysis to compare agreement rates between PATHWAY anti-HER2 (4B5) antibody and the VENTANA HER2 Dual ISH DNA Probe Cocktail assay, and between PATHWAY anti-HER2 (4B5) antibody and the PathVysion FISH assay. In this study IHC scores of 2+/3+ were considered positive for HER2 overexpression. Agreement data for the PathVysion HER-2 FISH assay and PATHWAY HER2/neu (4B5) antibody are shown in Table 9. Agreement data for the VENTANA HER2 Dual ISH DNA Probe Cocktail assay and PATHWAY HER2/neu (4B5) antibody are shown in Table 10.

Table 9. IHC on the BenchMark ULTRA instrument vs. FISH Comparison: Pooled Data from All Sites.

		PathVysion HER-2 FISH Result		
		Amplified	Non-Amplified	Total
PATHWAY HER2 (4B5) antibody Results	Positive (2+/3+ cases)	277	63	340
	Negative (0/1+)	27	238	265
	Total	304	301	605

	n/N	% (95% Score CI)
Positive Percent Agreement	277/304	91.1 (87.4, 93.8)

Table 10. IHC on the BenchMark ULTRA instrument vs. VENTANA HER2 Dual ISH DNA Probe Cocktail assay Comparison: Pooled Data from All Sites.

		VENTANA HER2 Dual ISH DNA Probe Cocktail Result		
		Amplified	Non-Amplified	Total
PATHWAY HER2 (4B5) antibody Results	Positive (2+/3+ cases)	248	78	326
	Negative (0/1+)	18	253	271
	Total	266	331	597

	n/N	% (95% Score CI)
Positive Percent Agreement	248/266	93.2 (89.6, 95.7)

Concordance Study: VENTANA HER2 Dual ISH DNA Probe Cocktail assay on BenchMark ULTRA instrument vs. Dako HER2 IQFISH pharmDx™ Kit assay

A concordance study was performed to evaluate the VENTANA HER2 Dual ISH DNA Probe Cocktail assay compared to Dako HER2 IQFISH pharmDx™ Kit for fluorescent in situ hybridization (FISH) in determination of *HER2* gene status in GEA. Comparability of the assay on GEA specimens was determined by comparing the staining results from the two assays (Table 11). One hundred thirty-four (134) human GEA specimens (a mix of amplified and non-amplified cases) were stained using VENTANA HER2 Dual ISH DNA Probe Cocktail. The same cohort was stained using the Dako HER2 IQFISH pharmDx™ assay. The results detailing negative, positive and overall agreement rates for the 146 samples of this cohort that were enumerable with both the Dako HER2 IQFISH pharmDx™ assay and the VENTANA HER2 Dual ISH DNA Probe Cocktail assay are shown in Table 11 and Table 12.

Table 11. Agreement between VENTANA HER2 Dual ISH DNA Probe Cocktail and the Dako HER2 IQFISH pharmDx™ assay in a cohort of human GEA specimens.

VENTANA HER2 Dual ISH DNA Probe Cocktail Amplification Status	Dako HER2 IQFISH pharmDx™ assay Amplification Status	
	Amp	Non-Amp
Amp	49	8
Non-Amp	5	84

Table 12. Summary of negative, positive, and overall agreement rates for VENTANA HER2 Dual ISH DNA Probe Cocktail and Dako HER2 IQFISH pharmDx™ on human GEA specimens.

	Negative Agreement Rate		Positive Agreement Rate		Overall Agreement Rate	
	Raw Data / # of Cases	Percent (95% Score CI)	Raw Data / # of Cases	Percent (95% Score CI)	Raw Data / # of Cases	Percent (95% Score CI)
VENTANA HER2 Dual ISH DNA Probe Cocktail	84/92	91.3 (83.8 – 95.5)	49/54	90.7 (80.1 – 96.0)	133/146	91.1 (85.4 – 94.7)

ANALYTICAL PERFORMANCE

BenchMark IHC/ISH instrument Repeatability and Precision with Breast Carcinoma

The repeatability and precision of VENTANA HER2 Dual ISH DNA Probe Cocktail were evaluated on BenchMark IHC/ISH instruments in combination with the VENTANA Silver ISH DNP Detection Kit and VENTANA Red ISH DIG Detection Kit.

Within-Run Repeatability was evaluated using 28 breast carcinoma specimens. Two replicate slides from each specimen were stained with VENTANA HER2 Dual ISH DNA Probe Cocktail on a single BenchMark ULTRA, BenchMark XT, or BenchMark GX instrument. For the analysis of the BenchMark XT and GX instrument data, the cases with ratios between 1.5 to 2.5 were weighted to their prevalence.

Between-Day Intermediate Precision was also evaluated using breast carcinoma specimens. Replicate slides from each of the 28 specimens were stained with VENTANA HER2 Dual ISH DNA Probe Cocktail on BenchMark IHC/ISH instruments on 5 non-consecutive days. For the analysis of the BenchMark XT and BenchMark GX instrument data, the cases with ratios between 1.5 to 2.5 were weighted to their prevalence.

Within-Run Repeatability was determined with average positive agreement (APA), average negative agreement (ANA), and overall percent agreement (OPA). The Between-Day Intermediate Precision was determined with positive percent agreement (PPA), negative percent agreement (NPA), and overall percentage agreement (OPA) across all the observations from the evaluable population. A summary of the results of both studies can be found in Table 13.

Table 13. BenchMark IHC/ISH Instrument Within-Run Repeatability and Between-Day Intermediate Precision.

Platform	Repeatability / Precision	Clinical Status	Agreement			
			Type	n/N	%	95% CI
ULTRA	Within-Run Repeatability	Amplified	APA	194/194	100	(98.1, 100)
		Non-Amplified	ANA	186/186	100	(98.0, 100)
		Total	OPA	190/190	100	(98.0, 100)
ULTRA	Between-Day Intermediate Precision	Amplified	PPA	139/139	100	(97.3, 100)
		Non-Amplified	NPA	135/135	100	(97.2, 100)
		Total	OPA	274/274	100	(98.6, 100)
XT	Within-Run Repeatability	Amplified	APA	128.8/ 128.8	100	(97.1, 100)
		Non-Amplified	ANA	151.2/ 151.2	100	(97.5, 100)
		Total	OPA	140.0/ 140.0	100	(97.3, 100)
XT	Between-Day	Amplified	PPA	128.8/ 128.8	100	(97.1, 100)

Platform	Repeatability / Precision	Clinical Status	Agreement			
			Type	n/N	%	95% CI
	Intermediate Precision	Non-Amplified	NPA	151.2/ 151.2	100	(97.5, 100)
		Total	OPA	280.0/ 280.0	100	(98.6, 100)
GX	Within-Run Repeatability	Amplified	APA	128.8/ 128.8	100	(97.1, 100)
		Non-Amplified	ANA	151.2/ 151.2	100	(97.5, 100)
		Total	OPA	140.0/ 140.0	100	(97.3, 100)
GX	Between-Day Intermediate Precision	Amplified	PPA	128.8/ 128.8	100	(97.1, 100)
		Non-Amplified	NPA	151.2/ 151.2	100	(97.5, 100)
		Total	OPA	280.0/ 280.0	100	(98.6, 100)

Note: 95% CIs were calculated using the percentile bootstrap method; in instances where the point estimate was 100%, Wilson Score method was used. . Four cases with ISH ratios between 1.5 to 2.5, inclusive, were included in the study.

Between-Instrument Intermediate Precision with Breast Carcinoma

BenchMark IHC/ISH instrument between-instrument intermediate precision of the VENTANA HER2 Dual ISH DNA Probe Cocktail was determined by staining replicate slides of 28 breast carcinoma specimens on 3 BenchMark IHC/ISH instruments with the VENTANA HER2 Dual ISH DNA Probe Cocktail using the VENTANA Silver ISH DNP Detection Kit and VENTANA Red ISH DIG Detection Kit. The between-instrument intermediate precision was determined with PPA, NPA, and OPA across all the observations from the evaluable population. The cases with ratios between 1.5 to 2.5 were weighted to their prevalence (BenchMark XT / BenchMark GX instruments). A summary of the results of this study can be found in Table 14.

Table 14. BenchMark IHC/ISH Between-Instrument Intermediate Precision.

Platform	Precision	Clinical Status	Agreement			
			Type	n/N	%	95% CI
ULTRA	Between-Instrument Intermediate Precision	Amplified	PPA	84/84	100	(95.6, 100)
		Non-Amplified	NPA	84/84	100	(95.6, 100)
		Total	OPA	168/168	100	(97.8, 100)
XT	Between-Instrument Intermediate Precision	Amplified	PPA	77.3/ 77.3	100	(95.3, 100)
		Non-Amplified	NPA	90.7/ 90.7	100	(95.9, 100)
		Total	OPA	168.0/ 168.0	100	(97.8, 100)
GX	Between-Instrument Intermediate Precision	Amplified	PPA	76.2/ 76.2	100	(95.2, 100)
		Non-Amplified	NPA	90.7/ 90.7	100	(95.9, 100)
		Total	OPA	166.9/ 166.9	100	(97.8, 100)

Note: 95% CIs were calculated using the percentile bootstrap method; in instances where the point estimate was 100%, Wilson Score method was used. Four cases with ISH ratios between 1.5 to 2.5, inclusive, were included in the study.

Within-Reader and Between-Reader Precision with Breast Carcinoma

BenchMark IHC/ISH instrument within-reader and between-reader precision of the VENTANA HER2 Dual ISH DNA Probe Cocktail was determined by having three readers evaluate 60 breast carcinoma specimens stained with the VENTANA HER2 Dual ISH DNA Probe Cocktail using the VENTANA Silver ISH DNP Detection Kit and VENTANA Red ISH DIG Detection Kit on BenchMark ULTRA instrument. For within-reader precision, the same set of slides were read twice after a minimum of two weeks between reads. The within-reader and between-reader precision was determined with APA, ANA, and OPA across all the observations from the evaluable population. A summary of the results of this study can be found in Table 15.

Table 15. BenchMark ULTRA Instrument Within-Reader and Between-Reader Precision.

Precision	Clinical Status	Agreement			
		Type	n/N	%	95% CI
Within-Reader	Amplified	APA	178/181	98.3	(96.3, 100)
	Non-Amplified	ANA	174/177	98.3	(96.1, 100)
	Total	OPA	176/179	98.3	(96.1, 100)
Between Reader	Amplified	APA	350/362	96.7	(93.2, 99.4)
	Non-Amplified	ANA	342/354	96.6	(92.8, 99.4)
	Total	OPA	346/358	96.6	(92.8, 99.4)

Note: 95% CIs were calculated using the percentile bootstrap method. Six cases with ISH ratios between 1.5 to 2.5, inclusive, were included in the study.

Between Platform Precision with Breast Carcinoma

BenchMark IHC/ISH instrument between platform precision of the VENTANA HER2 Dual ISH DNA Probe Cocktail was determined by evaluating 28 breast carcinoma specimens stained with the VENTANA HER2 Dual ISH DNA Probe Cocktail using the VENTANA Silver ISH DNP Detection Kit and VENTANA Red ISH DIG Detection Kit on BenchMark IHC/ISH instruments. The between platform precision was determined with PPA, NPA, and OPA across all the observations from the evaluable population. The cases with ratios between 1.5 to 2.5 were weighted to their prevalence. A summary of the results of this study can be found in Table 16.

Table 16. BenchMark IHC/ISH Instrument Between Platform Precision.

Precision	Clinical Status	Agreement			
		Type	n/N	%	95% CI
Between Platform Precision	Amplified	PPA	230.8/ 230.8	100	(98.4, 100)
	Non-Amplified	NPA	271.0/ 272.2	99.6	(98.3, 100)
	Total	OPA	501.8/ 502.9	99.8	(99.2, 100)

Note: 95% CIs were calculated using the percentile bootstrap method; in instances where the point estimate was 100%, Wilson Score method was used. Four cases with ISH ratios between 1.5 to 2.5, inclusive, were included in the study.

BenchMark IHC/ISH Instrument: Repeatability and Precision with Gastric Adenocarcinoma

The repeatability and precision of VENTANA HER2 Dual ISH DNA Probe Cocktail were evaluated on the BenchMark IHC/ISH instruments in combination with the VENTANA Silver ISH DNP Detection Kit and VENTANA Red ISH DIG Detection Kit.

Within-Run Repeatability was evaluated using fourteen gastric adenocarcinoma specimens. Two replicate slides from each of the gastric adenocarcinoma specimens were stained with VENTANA HER2 Dual ISH DNA Probe Cocktail on a single BenchMark IHC/ISH instrument. The cases with ratios between 1.5 to 2.5 were weighted to their prevalence.

Between-Day Intermediate Precision was also evaluated using gastric adenocarcinoma specimens. Replicate slides from each of the fourteen specimens were stained with VENTANA HER2 Dual ISH DNA Probe Cocktail on BenchMark IHC/ISH instruments on 5 non-consecutive days. The cases with ratios between 1.5 to 2.5 were weighted to their prevalence.

Within-Run Repeatability was determined with average positive agreement (APA), average negative agreement (ANA), and overall percent agreement (OPA). The Between-Day Intermediate Precision was determined with positive percent agreement (PPA), negative percent agreement (NPA), and overall percentage agreement (OPA) across all the observations from the evaluable population. A summary of the results of both studies can be found in Table 17.

Table 17. BenchMark IHC/ISH Instrument: Within-Run Repeatability and Between-Day Intermediate Precision.

Platform	Repeatability / Precision	Clinical Status	Agreement			
			Type	n/N	%	95% CI
ULTRA	Within-Run Repeatability	Amplified	APA	70.0/ 70.0	100	(94.8, 100)
		Non-Amplified	ANA	70.0/ 70.0	100	(94.8, 100)
		Total	OPA	70.0/ 70.0	100	(94.8, 100)
ULTRA	Between-Day Intermediate Precision	Amplified	PPA	70.0/ 70.0	100	(94.8, 100)
		Non-Amplified	NPA	70.0/ 70.0	100	(94.8, 100)
		Total	OPA	140.0/ 140.0	100	(97.3, 100)
XT	Within-Run Repeatability	Amplified	APA	70.0/ 70.0	100	(94.8, 100)
		Non-Amplified	ANA	70.0/ 70.0	100	(94.8, 100)
		Total	OPA	70.0/ 70.0	100	(94.8, 100)
XT	Between-Day Intermediate Precision	Amplified	PPA	70.0/ 70.0	100	(94.8, 100)
		Non-Amplified	NPA	70.0/ 70.0	100	(94.8, 100)
		Total	OPA	140.0/ 140.0	100	(97.3, 100)
GX	Within-Run Repeatability	Amplified	APA	64.6/ 65.1	99.1	(95.9, 100)
		Non-Amplified	ANA	70.0/ 70.6	99.2	(95.2, 100)
		Total	OPA	67.3/ 67.9	99.2	(96.9, 100)
GX	Between-Day Intermediate Precision	Amplified	PPA	67.3/ 67.9	99.2	(96.5, 100)
		Non-Amplified	NPA	70.0/ 70.0	100	(94.8, 100)
		Total	OPA	137.3/ 137.9	99.6	(98.5, 100)

Note: 95% CIs were calculated using the percentile bootstrap method; in instances where the point estimate was 100%, Wilson Score method was used. Two cases with ISH ratios between 1.5 to 2.5, inclusive, were included in the study.

Between-Instrument Intermediate Precision with Gastric Adenocarcinoma

BenchMark IHC/ISH instrument between-instrument intermediate precision of the VENTANA HER2 Dual ISH DNA Probe Cocktail was determined by staining replicate slides of fourteen gastric adenocarcinoma specimens on 3 BenchMark IHC/ISH instruments with the VENTANA HER2 Dual ISH DNA Probe Cocktail using the VENTANA Silver ISH DNP Detection Kit and VENTANA Red ISH DIG Detection Kit. The between-instrument intermediate precision was determined with PPA, NPA, and OPA across all the observations from the evaluable population. The cases with ratios between 1.5 to 2.5 were weighted to their prevalence. A summary of the results of this study can be found in Table 18.

Table 18. BenchMark IHC/ISH Between-Instrument Intermediate Precision.

Platform	Precision	Clinical Status	Agreement			
			Type	n/N	%	95% CI
ULTRA	Between-Instrument Intermediate Precision	Amplified	PPA	42.0/42.0	100	(91.6, 100)
		Non-Amplified	NPA	42.0/42.0	100	(91.6, 100)
		Total	OPA	84.0/84.0	100	(95.6, 100)
XT	Between-Instrument Intermediate Precision	Amplified	PPA	40.4/40.9	98.6	(94.1, 100)
		Non-Amplified	NPA	40.9/40.9	100	(91.4, 100)
		Total	OPA	81.3/81.9	99.3	(97.5, 100)
GX	Between-Instrument Intermediate Precision	Amplified	PPA	40.9/40.9	100	(91.4, 100)
		Non-Amplified	NPA	42.0/42.0	100	(91.6, 100)
		Total	OPA	82.9/82.9	100	(95.6, 100)

Note: 95% CIs were calculated using the percentile bootstrap method; in instances where the point estimate was 100%, Wilson Score method was used. Two cases with ISH ratios between 1.5 to 2.5, inclusive, were included in the study.

Within-Reader and Between-Reader Precision with Gastric Adenocarcinoma

BenchMark IHC/ISH instrument within-reader and between-reader precision of the VENTANA HER2 Dual ISH DNA Probe Cocktail was determined by having three readers evaluate 28 gastric adenocarcinoma specimens stained with the VENTANA HER2 Dual ISH DNA Probe Cocktail using the VENTANA Silver ISH DNP Detection Kit and VENTANA Red ISH DIG Detection Kit on BenchMark ULTRA instrument. All slides were randomized and masked to the case diagnosis. For within-reader precision, the same set of slides were read twice after a minimum of two weeks between reads. The within-reader precision and between-reader precision was determined with APA, ANA, and OPA across all the observations from the evaluable population. A summary of the results of this study can be found in Table 19.

Table 19. BenchMark ULTRA Instrument Within-Reader and Between-Reader Precision.

Precision	Clinical Status	Agreement			
		Type	n/N	%	95% CI
Between Reader	Amplified	APA	80/84	95.2	(90.5, 100)
	Non-Amplified	ANA	80/84	95.2	(90.5, 100)
	Total	OPA	80/84	95.2	(90.5, 100)
Within-Reader	Amplified	APA	82/84	97.6	(95.2, 100)
	Non-Amplified	ANA	82/84	97.6	(95.2, 100)
	Total	OPA	82/84	97.6	(95.2, 100)

Note: 95% CIs were calculated using the percentile bootstrap method. Two cases with ISH ratios between 1.5 to 2.5, inclusive, were included in the study.

Between Platform Precision with Gastric Adenocarcinoma

BenchMark IHC/ISH instrument between platform precision of the VENTANA HER2 Dual ISH DNA Probe Cocktail was determined by evaluating fourteen gastric adenocarcinoma specimens stained with the VENTANA HER2 Dual ISH DNA Probe Cocktail using the VENTANA Silver ISH DNP Detection Kit and VENTANA Red ISH DIG Detection Kit on BenchMark IHC/ISH instruments. The between platform precision was determined with PPA, NPA, and OPA across all the observations from the evaluable population. The cases with ratios between 1.5 to 2.5 were weighted to their prevalence. A summary of the results of this study can be found in Table 20.

Table 20. BenchMark IHC/ISH Instrument: Between Platform Precision.

Precision	Clinical Status	Agreement			
		Type	n/N	%	95% CI
Between Platform Precision	Amplified	PPA	123.3/123.9	99.5	(98.1, 100)
	Non-Amplified	NPA	124.9/124.9	100	(97.0, 100)
	Total	OPA	248.2/248.8	99.8	(99.2, 100)

Note: 95% CIs were calculated using the percentile bootstrap method; in instances where the point estimate was 100%, Wilson Score method was used. Two cases with ISH ratios between 1.5 to 2.5, inclusive, were included in the study.

Lot-to-Lot Precision with Breast Carcinoma

Lot-to-Lot Precision was determined by testing 3 production lots of the VENTANA HER2 Dual ISH DNA Probe Cocktail, VENTANA Silver ISH DNP Detection Kit, and VENTANA Red ISH DIG Detection Kit on BenchMark ULTRA instruments. Twenty-eight breast carcinoma cases were stained with each probe and detection kit. A summary of the results for Lot-to-Lot Precision of the assay is shown in Table 21.

Table 21. Lot-to-Lot Precision.

Precision	Clinical Status	Agreement			
		Type	n/N	%	95% CI
Lot-to-Lot	Amplified	PPA	121/121	100	(96.9, 100)
	Non-Amplified	NPA	123/123	100	(97.0, 100)
	Total	OPA	244/244	100	(98.5, 100)

Note: 95% CIs were calculated using the percentile bootstrap method; in instances where the point estimate was 100%, Wilson Score method was used. Four cases with ISH ratios between 1.5 to 2.5, inclusive, were included in the study.

BenchMark ULTRA Instrument Inter-laboratory Reproducibility Study with Breast Carcinoma and Gastric Adenocarcinoma

An inter-laboratory reproducibility (ILR) study was conducted to evaluate the reproducibility of VENTANA HER2 Dual ISH DNA Probe Cocktail to determine *HER2* gene status in breast carcinoma and gastric adenocarcinoma tissue stained on the BenchMark ULTRA instrument in combination with the VENTANA Silver ISH DNP Detection Kit and VENTANA Red ISH DIG Detection Kit.

Twenty-eight FFPE breast and gastric adenocarcinoma tissue specimens were used and approximately half of these cases were amplified for *HER2* expression status and half were non-amplified for *HER2* status.

Multiple tissue sections were cut from each specimen and provided to 3 external study sites. Each site stained 28 breast and 28 gastric cases on each of 5 non-consecutive days over a minimum of 20 days. Following staining on the BenchMark ULTRA instrument a reader evaluated each slide to assign *HER2* gene status.

The results of the study are summarized in Table 22 and Table 23, below. The data was analyzed for PPA and NPA across all observations. For each case, all evaluable observations (amplified vs non-amplified) were compared against the modal result for each case. The cases with ratios between 1.5 to 2.5 were weighted to their prevalence. These comparisons were pooled across sites and days, and then results were aggregated across cases.

Table 22. ILR: Agreement Rates on the BenchMark ULTRA instrument for Breast Carcinoma.

Inter-Laboratory Reproducibility		Agreement			
		Type	n/N	%	95% CI
Between-Site (3 sites)		PPA	208.9/208.9	100	(98.2, 100.0)
		NPA	198.1/200.3	98.9	(96.8, 100.0)
		OPA	407.0/409.3	99.5	(98.4, 100.0)
Between-Day (5 non-consecutive days)	Site A	PPA	72/74	97.3	(92.3, 100.0)
		NPA	63/63	100	(94.3, 100.0)
		OPA	135/137	98.5	(95.6, 100.0)
	Site B	PPA	70/70	100	(94.8, 100.0)
		NPA	63/64	98.4	(95.8, 100.0)
		OPA	133/134	99.3	(97.8, 100.0)
	Site C	PPA	70/70	100	(94.8, 100.0)
		NPA	69/69	100	(94.7, 100.0)
		OPA	139/139	100	(97.3, 100.0)

Note: 95% CIs were calculated using the percentile bootstrap method; in instances where the point estimate was 100%, Wilson Score method was used. Four cases with ISH ratios between 1.5 to 2.5, inclusive, were included in the study.

Table 23. ILR: Agreement Rates on the BenchMark ULTRA instrument for Gastric Adenocarcinoma.

Inter-Laboratory Reproducibility		Agreement			
		Type	n/N	%	95% CI
Between-Site (3 sites)		PPA	206.8/206.8	100	(98.2, 100.0)
		NPA	208.4/208.9	99.7	(99.2, 100.0)
		OPA	415.1/415.7	99.9	(99.6, 100.0)
Between-Day (5 non-consecutive days)	Site A	PPA	70/70	100	(94.8, 100.0)
		NPA	69/70	98.6	(96.0, 100.0)
		OPA	139/140	99.3	(97.9, 100.0)
	Site B	PPA	67/67	100	(94.6, 100.0)
		NPA	69/69	100	(94.7, 100.0)
		OPA	136/136	100	(97.3, 100.0)
	Site C	PPA	70/70	100	(94.8, 100.0)
		NPA	70/70	100	(94.8, 100.0)
		OPA	140/140	100	(97.3, 100.0)

Note: 95% CIs were calculated using the percentile bootstrap method; in instances where the point estimate was 100%, Wilson Score method was used. Four cases with ISH ratios between 1.5 to 2.5, inclusive, were included in the study.

Performance of VENTANA HER2 Dual ISH DNA Probe Cocktail on the BenchMark ULTRA PLUS Instrument

Concordance Between BenchMark ULTRA PLUS and BenchMark ULTRA Instruments for Breast Carcinoma

Three laboratories, from separate institutions in the United States, participated in a concordance study between the BenchMark ULTRA PLUS instrument versus the BenchMark ULTRA instrument. There were 193 unique FFPE invasive breast carcinoma cases which represented the staining range of the VENTANA HER2 Dual ISH Cocktail assay, with approximately equal distribution between *HER2*-amplified and *HER2*–non-amplified cases. Tissue slides from all cases were stained with H&E as well as VENTANA HER2 Dual ISH Cocktail by Roche on a BenchMark ULTRA instrument using the recommended staining protocol. Unstained tissue slides from all cases were randomized and equally distributed (64-65 cases/per site) across study sites for staining on a BenchMark ULTRA PLUS instrument using the recommended VENTANA HER2 Dual ISH Cocktail staining protocol. Pathologists, blinded to case status, evaluated the slides stained on one BenchMark IHC/ISH instrument and determined the *HER2* gene status. After a two week period, pathologists evaluated the slides stained on the second BenchMark IHC/ISH instrument. *HER2* gene status was determined using the ratio of *HER2* gene signals to chromosome 17 (Chr17) signals (i.e., the *HER2*:Chr17 ratio) in the tumor cell nuclei. If the ratio was 2.0 or greater, the case was considered *HER2*-amplified; if it was less than 2.0, it was considered *HER2*–non-amplified. The results were analyzed by Roche. The OPA, PPA and NPA rates were 97.1% (535/551), 97.3% (248/255), and 97.0% (287/296), respectively. The results are summarized in Table 24.

Table 24. Pooled Agreement of *HER2* Gene Status for Breast Carcinoma Cases Stained with VENTANA HER2 Dual ISH Cocktail on the BenchMark ULTRA PLUS vs BenchMark ULTRA Instrument

BenchMark ULTRA PLUS *HER2* Gene Status	BenchMark ULTRA *HER2* Gene Status		Total
	Amplified	Non-Amplified	
Amplified	248	9	257
Non-Amplified	7	287	294
Total	255	296	551
	n/N	% (95% CI)	
PPA	248/255	97.3 (95.0, 99.2)	
NPA	287/296	97.0 (94.8, 99.0)	
OPA	535/551	97.1 (95.5, 98.6)	

Note: Two-sided 95% CIs were calculated using the percentile bootstrap method with 2000 replicates selected with stratification on the 4 diagnostic score bins used during case selection [amplified (not borderline), non-amplified (not borderline), borderline amplified, borderline non-amplified]

BenchMark ULTRA PLUS Instrument Inter-laboratory Reproducibility Study with Breast Carcinoma

An inter-laboratory reproducibility (ILR) study was conducted to evaluate the reproducibility of VENTANA HER2 Dual ISH DNA Probe Cocktail to determine *HER2* gene status in breast carcinoma tissue stained on the BenchMark ULTRA PLUS instrument in combination with the VENTANA Silver ISH DNP Detection Kit and VENTANA Red ISH DIG Detection Kit.

Twenty-eight unique FFPE invasive breast carcinoma cases, which represented the staining range of the VENTANA HER2 Dual ISH Cocktail assay, were used with an approximate equal distribution between *HER2*-amplified and *HER2*–non-amplified cases.

Multiple tissue sections were cut from each specimen and provided to 3 external study sites. All 28 cases were stained on a BenchMark ULTRA PLUS instrument on each of 5 non-consecutive days over a minimum of 20 days at each site. Readers evaluated the slides and determined the *HER2* gene status.

Results are summarized in Table 25 and Table 26. The data was analyzed for PPA, NPA and OPA in Table 25 and APA, ANA, OPA in Table 26 across all observations. For each case, all evaluable observations (amplified vs non-amplified) were compared against the modal result for each case. These comparisons were pooled across sites and days, and then results were aggregated across cases.

Table 25. ILR: Agreement Rates with Modal Status on the BenchMark ULTRA PLUS instrument for Breast Carcinoma.

Inter-Laboratory Reproducibility	Agreement			
	Type	n/N	%	95% CI
Overall	PPA	372/381	97.6	(95.3, 100.0)
	NPA	421/440	95.7	(91.1, 99.3)
	OPA	793/821	96.6	(94.3, 98.5)
Between-Site (3 sites)	PPA	380/389	97.7	(95.3, 100.0)
	NPA	421/432	97.5	(95.3, 99.3)
	OPA	801/821	97.6	(96.3, 98.7)
Between-Reader	PPA	383/389	98.5	(97.1, 99.5)
	NPA	424/432	98.1	(97.1, 99.0)
	OPA	807/821	98.3	(97.5, 99.0)

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

Table 26. ILR: Pairwise Agreement Rates on the BenchMark ULTRA PLUS instrument for Breast Carcinoma

Inter-Laboratory Reproducibility	Agreement			
	Type	n/N	%	95% CI
Between-Site (3 sites)	APA	7204/7652	94.1	(91.1, 96.9)
	ANA	7968/8416	94.7	(91.5, 97.4)
	OPA	7586/8034	94.4	(91.5, 97.1)
Between-Reader	APA	370/390	94.9	(92.5, 97.1)
	ANA	408/428	95.3	(92.7, 97.5)
	OPA	389/409	95.1	(92.7, 97.3)
Between-Day (5 non-consecutive days)	APA	1472/1519	96.9	(95.5, 98.2)
	ANA	1642/1689	97.2	(95.8, 98.5)
	OPA	1557/1604	97.1	(95.7, 98.3)

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

Concordance Between BenchMark ULTRA PLUS and BenchMark ULTRA Instruments for Gastric Carcinoma

There were 109 unique FFPE invasive gastric carcinoma cases which represented the staining range of the VENTANA HER2 Dual ISH Cocktail assay, with approximately equal distribution between *HER2*-amplified and *HER2*–non-amplified cases. Tissue slides were stained on a BenchMark ULTRA PLUS instrument and BenchMark ULTRA instrument using the recommended staining protocol. Stained slides were scored by a pathologist. The overall percent agreement for VENTANA HER2 Dual ISH Cocktail staining based on *HER2* gene status (*HER2*-amplified, *HER2*-non-amplified) was 92.4%. Two-sided 95% confidence intervals, which is 84.4% to 96.5%, were calculated using the Wilson score method. Background and morphology acceptability rates for all cases were 100% for the BenchMark ULTRA PLUS instrument.

BenchMark ULTRA PLUS Instrument Precision Studies for Gastric Carcinoma

Twelve gastric carcinoma tissue cases, which represented the staining range of the VENTANA HER2 Dual ISH Cocktail assay, were tested on the BenchMark ULTRA PLUS instrument. The cases had approximately equal distribution between *HER2*-amplified and *HER2*–non-amplified *HER2* gene status. Stained slides were evaluated by a pathologist.

All agreement rates were calculated using a two-sided 95% confidence intervals using the percentile bootstrap method.

For Within-Run Repeatability, five slides per case were stained on the BenchMark ULTRA PLUS instrument. The overall percent agreement for VENTANA HER2 Dual ISH Cocktail staining, based on *HER2* gene status (*HER2*-amplified, *HER2*-non-amplified), was 91.7% (95% CI: 81.7, 100.0).

For Between-Day Intermediate Precision, two slides per case were stained on the BenchMark ULTRA PLUS instrument in five staining runs conducted over a five, non-consecutive day period. The overall percent agreement for VENTANA HER2 Dual ISH Cocktail staining based on *HER2* gene status (*HER2*-amplified, *HER2*-non-amplified) was 90.8% (95% CI: 80.8, 100.0)

For Between-Instrument Intermediate Precision, two slides per case were stained on each of three BenchMark ULTRA PLUS instruments. The overall percent agreement for VENTANA HER2 Dual ISH Cocktail staining based on *HER2* gene status (*HER2*-amplified, *HER2*-non-amplified) was 92.6% (95% CI: 84.5, 100.0).

Sensitivity and Specificity

Analytical specificity (hybridization efficacy) of the VENTANA HER2 Dual ISH DNA Probe Cocktail assay was determined by staining normal human metaphase spreads on a BenchMark ULTRA instrument. Of 100 metaphase spreads analyzed, 100% exhibited specific co-localization of both HER2 and Chromosome 17 probes.

Analytical sensitivity measures the ability of the probe to detect its specific target, while specificity is its ability to distinguish the target from other sequences in the specimen. The assay has an analytical sensitivity and specificity control built into each human tissue. Normal human cells (including: stromal fibroblasts, endothelial cells, lymphocytes, and non-neoplastic breast epithelial cells) should contain 1-2 copies of *HER2* and Chr17. Therefore, 1-2 copies for *HER2* and Chr17 in normal human cells indicates that the probes are detecting their specific target (a measure of sensitivity). One to two copies for *HER2* and Chr17 in normal cells also indicates that the probe is detecting only its specific targets (a measure of specificity). The first pass rate for the VENTANA HER2 Dual ISH DNA Probe Cocktail assay on 40 breast samples fixed within the ASCO CAP guidelines (10% NBF for 6 to 72 hours) was 97.5% (87.1– 99.6) on BenchMark ULTRA instruments, 100% (91.2 – 100) on BenchMark XT instruments, and 97.5% (87.1 – 99.6) on BenchMark GX instruments. Specificity on the same 40 breast samples with no probe control was 100% (91.2 – 100) on BenchMark ULTRA instruments.

The first pass rate for the VENTANA HER2 Dual ISH DNA Probe Cocktail assay on 39 gastric samples fixed within the ASCO CAP guidelines (10% NBF for 6 to 72 hours) was 97.4% (86.8 – 99.5) on BenchMark ULTRA instruments, 97.4% (86.8 – 99.5) on BenchMark XT instruments, and 100% (91 – 100) on BenchMark GX instruments.

Analytical sensitivity and specificity was also assessed by staining multiple cases of normal and neoplastic human tissues with VENTANA HER2 Dual ISH DNA Probe Cocktail assay, VENTANA Silver ISH DNP Detection Kit, and VENTANA Red ISH DIG Detection Kit. The results are listed in Table 27 and Table 28. No unexpected staining was observed with VENTANA HER2 Dual ISH DNA Probe Cocktail assay on the normal and neoplastic tissues.

Table 27. Sensitivity/Specificity of VENTANA HER2 Dual ISH DNA Probe Cocktail assay was determined by testing FFPE normal tissues.

Tissue	# acceptable / total cases	Tissue	# acceptable / total cases
Adrenal gland	3/3	Lung	3/3
Bladder	3/3	Lymph node	3/3
Bone marrow	3/3	Mesothelium	3/3
Ovary	3/3	Pancreas	3/3
Breast	3/3	Parathyroid gland	3/3
Cerebellum	3/3	Peripheral nerve	3/3
Cerebrum	3/3	Prostate	3/3
Cervix	3/3	Skeletal Muscle	3/3
Colon	3/3	Skin	3/3

Tissue	# acceptable / total cases	Tissue	# acceptable / total cases
Endometrium	3/3	Spleen	3/3
Esophagus	3/3	Stomach	3/3
Heart	3/3	Testis	3/3
Hypophysis (Pituitary)	3/3	Thymus	3/3
Intestine	3/3	Thyroid	3/3
Kidney	3/3	Tongue/Salivary gland	3/3
Liver	3/3	Tonsil	3/3

Table 28. Sensitivity/Specificity of VENTANA HER2 Dual ISH DNA Probe Cocktail assay was determined by testing a variety of FFPE neoplastic tissues.

Pathology	# acceptable / total cases
Glioblastoma (Cerebrum)	3/3
Meningioma (Cerebrum)	1/1
Oligodendroglioma (Cerebrum)	1/1
Endometrioid Carcinoma (Ovary)	1/1
Adenocarcinoma (Ovary)	1/1
Pancreatic neuroendocrine neoplasm (Pancreas)	1/1
Adenocarcinoma (Pancreas)	1/1
Seminoma (Testis)	1/1
Embryonal carcinoma (Testis)	1/1
Medullary carcinoma (Thyroid)	1/1
Papillary carcinoma (Thyroid)	1/1
Ductal carcinoma in situ (Breast)	1/1
Invasive ductal carcinoma (Breast)	2/2
B-cell lymphoma; NOS (Spleen)	1/1
Small cell carcinoma (Lung)	1/1
Squamous cell carcinoma (Lung)	1/1
Adenocarcinoma (Esophagus)	1/1
Squamous cell carcinoma (Esophagus)	1/1
Adenocarcinoma (Stomach)	1/1
Adenocarcinoma (Gastroesophageal junction)	1/1
Adenocarcinoma (Small intestine)	1/1
Gastrointestinal stromal tumor (GIST) (Small intestine)	1/1
Gastrointestinal stromal tumor (GIST) (Colon)	1/1
Adenocarcinoma (Colon)	1/1
Adenocarcinoma (Rectum)	1/1
Gastrointestinal stromal tumor (GIST) (Rectum)	1/1
Hepatoblastoma (Liver)	1/1
Hepatocellular carcinoma (Liver)	1/1
Clear cell carcinoma (Kidney)	1/1
Adenocarcinoma (Prostate)	2/2
Leiomyoma (Uterus)	1/1
Endometrioid adenocarcinoma (Uterus)	1/1
Clear cell carcinoma (Uterus)	1/1
Squamous cell carcinoma (Cervix)	2/2
Embryonal rhabdomyosarcoma (Striated muscle)	1/1
Squamous cell carcinoma (Skin)	1/1
Basal cell carcinoma (Skin)	1/1
Neurofibroma (Lumbar)	1/1
Neuroblastoma (Retroperitoneum)	1/1
Mesothelioma (Peritoneum)	1/1
B-cell lymphoma; NOS (Lymph node)	2/2
Hodgkin lymphoma (Lymph node)	3/3
Anaplastic large cell lymphoma (Lymph node)	1/1
Leiomyosarcoma (Bladder)	1/1
Urothelial carcinoma (Bladder)	1/1
Osteosarcoma (Bone)	1/1
Mesothelioma (Peritoneum)	1/1
Leiomyosarcoma (Smooth muscle)	1/1

TROUBLESHOOTING

Table 29. Troubleshooting Solutions.

Issue	Solution
Absent or Weak SISH Staining	1. Ensure reagent dispensers are functioning properly (i.e., not clogged or empty) and bulk solutions are filled. Check the reagent dispenser priming chamber or meniscus for foreign materials or particulates, such as fibers or precipitates. If the dispenser is blocked, do not use the dispenser and contact your local support representative. Otherwise, re-prime the dispenser by aiming the dispenser over a waste container, removing the nozzle cap, and pressing down on the top of the dispenser. If staining is still weak or absent, proceed to 2 below. 2. Ensure fixation type, time and section thickness is appropriate for ISH-based assays. 3. Ensure use of SISH compatible mounting media (see Table 30) to preserve SISH signal. If staining is still weak or absent, proceed to 4 below. 4. Increase CC1 time to > 16 min. 5. Increase CC2 time to > 16 min for gastric carcinoma/GEA or > 24 min for breast carcinoma. 6. Increase ISH Protease 3 time to > 16 min for gastric carcinoma or > 20 min for breast carcinoma if nuclear morphology is intact.

Issue	Solution
Absent or Weak Red ISH Staining	1. Ensure reagent dispensers are functioning properly (i.e., not clogged or empty) and bulk solutions are filled. If staining is still weak or absent, proceed to 2 below. 2. Ensure alcohol baths and extended xylene washes are not used to dehydrate stained slides, as this will degrade Red ISH signals. If staining is still weak or absent, proceed to 3 below. 3. Ensure fixation type, time and section thickness is appropriate for ISH-based assays. 4. Increase CC1 time to > 16 min. 5. Increase CC2 time to > 16 min for gastric carcinoma or > 24 min for breast carcinoma. 6. Increase ISH Protease 3 time to > 16 min for gastric carcinoma or > 20 min for breast carcinoma if nuclear morphology is intact.
Nonspecific Red ISH Background	1. Ensure that positively charged slides are used and specimen is fixed and sectioned appropriately for ISH-based assays. 2. If Red ISH background is discernible from specific Red ISH signal, enumerate the slide but do not count non-specific Red ISH signals. 3. If Red ISH background in the nucleus interferes with enumeration, repeat the staining using 76°C or 78°C stringency wash temperature. Decreasing protease or cell conditioning time also mitigates red background.
Nonspecific SISH Background	1. Ensure that positively charged slides are used and specimen is fixed and sectioned appropriately for ISH-based assays. 2. If SISH background is discernible from specific SISH signal, enumerate the slide but do not count non-specific signals. 3. If SISH background in the nucleus interferes with enumeration, repeat the staining with lower protease treatment or lower cell conditioning time.
Precipitation	1. If precipitation artifact interferes with enumeration, repeat the staining. If SISH background is discernible from specific SISH signal, enumerate the slide but do not count non-specific signals. 2. Ensure that barcode slide labels are centered and applied to the glass slide with no label overhang. Do not double label or reapply barcode labels.
Bubbling	1. If bubbling interferes with enumeration, ensure pre analytical procedures and sample thickness are as recommended.
Tissue washes off slides.	1. Ensure that positively-charged slides are used.

Table 30. Compatibility of Mounting Media with SISH based assays.

Mounting Media	Manufacturer	Type (Xylene, alcohol, aqueous)	Compatibility with SISH
Entellan	Merck	Xylene	No
Entellan New	Merck	Xylene	No
Eukitt	EMS	Xylene	No
HSR	Sysmex	Xylene	No
Malinol	Muto Chemical	Xylene	No
Acrytol	SurgiPath	Xylene	Yes
Alcolmount	Diapath	Alcohol	Yes
BioMount 2	BBInternational	Xylene	Yes
Cytoseal 60	Richard Allan Scientific	Xylene	Yes
Cytoseal XYL	Richard Allan Scientific	Xylene	Yes
Diamount	Diapath	Xylene	Yes
DPX	BDH: Raymond Lamb	Xylene	Yes
FloTexx	Lerner Labs	Xylene	Yes
Gel Mount	Biomeda	Aqueous	Yes
Histomount	Raymond Lamb	Xylene	Yes
MicroMount	SurgiPath	Xylene	Yes
MM24	SurgiPath	Xylene	Yes
Mountex	Histolab	Xylene	Yes
MountQuick	Daido Sangyo Co.	Aqueous	Yes
Paramount	Protaqs Quartett: Dako	Xylene	Yes
Permount	Fisher	Xylene	Yes
Pertex	Cell Path	Xylene	Yes
Shandon Consul mount	Thermo Scientific	Xylene	Yes
Softmount	WAKO	Lemasol A	Yes
SureMount	Triangle Biomedical Sciences	Xylene	Yes
Thermo EZ Mount	Thermo Scientific	Xylene	Yes
Ultramount	Dako	Xylene	Yes

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

The summary of safety and performance can be found here:

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

Symbols

Ventana uses the following symbols and signs in addition to those listed in the ISO 15223-1 standard (for USA: see elabdoc.roche.com/symbols for more information).

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

REVISION HISTORY

Rev	Updates
C	Updates to Warnings and Precautions and References Sections. Updated to current template.

INTELLECTUAL PROPERTY

For USA: Rx only

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CE

VENTANA®

Appendix A: VENTANA HER2 Dual ISH DNA Probe Cocktail Scoring Form

1. Case ID / Patient ID:	2. Is this case enumerable? 2a. ☐ *Yes-Proceed to #3* 2b. ☐ *No-Skip. #3. Proceed to #4*	3. Is tumor heterogeneity present? 3a. ☐ *Yes, Skip #4. Proceed to #5.* 3b. ☐ *No, Skip #4. Proceed to #5.*	
4. This case is not enumerable because (mark ALL that apply):	4a. ☐ *There was no tissue left on the ISH stained slide*	4b. ☐ *There was no invasive carcinoma in tissue on the ISH stained slide*	4c. ☐ *Nuclear Morphology is unacceptable; unable to distinguish tissue structural elements of normal cells from those of target carcinoma cells.*
			4d. ☐ *Background unacceptable, interferes with scoring ISH stained slide* ☐*HER2* ☐*Chr 17*
4e. ☐ *Internal Positive Control signal not detectable* ☐*HER2* ☐*Chr 17*	4f. ☐ *Weak/absent ISH staining in target cells, unable to score* ☐*HER2* ☐*Chr 17*	4g. ☐ *Other (specify):*	

5. Enumerate Target Area 1: Count HER2 signal and Chr17 signal in each of 20 nuclei. Add the HER2 signal counts. Add the Chr 17 counts. Create the gene status ratio by dividing the TOTAL HER2 signal count by the TOTAL Chr 17 signal count. Round to .1 decimal place. Document whether clusters of signals were counted.

SIGNAL COUNT TARGET AREA 1 – 20 nuclei

	5a	5b	5c	5d	5e	5f	5g	5h	5i	5j	5k	5l	5m	5n	5o	5p	5q	5r	5s	5t	5u	5v	5w	5x Comments
	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	TOTAL	RATIO	Clusters Present?	
HER2																							☐ Yes ☐ No	
Chr17																							☐ Yes ☐ No	

6. Result from 20 nuclei: 6a. ☐ Non- amplified: HER2/Chr17 < 2.0 or 6b. ☐ Amplified: HER2/Chr17 ≥ 2.0

If the HER2/Chr17 ratio falls between 1.8 and 2.2 then 20 additional nuclei should be enumerated.

7. Enumerate Target Area 2: Count HER2 signal and Chr17 signal in each of 20 nuclei. Add the HER2 signal counts. Add the Chr 17 counts. Document whether clusters of signals were counted.

. SIGNAL COUNT TARGET AREA 2 – 2nd 20 nuclei (if target area 1 ratio is 1.8 – 2.2)

	7a	7b	7c	7d	7e	7f	7g	7h	7i	7j	7k	7l	7m	7n	7o	7p	7q	7r	7s	7t	7u	8w	8x Comments
	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	TOTAL	Clusters Present?	
HER2																						☐ Yes ☐ No	
Chr17																						☐ Yes ☐ No	

8. Result from all 40 nuclei:

8a. Target Area 1 HER2 Total _____ (from box 5u) + Target Area 2 HER2 Total _____ (from box 7u) = Total HER2 count _____ 8c. Ratio: Total HER2 / Total Chr17

8b. Target Area 1 Chr17 Total _____ (from box 5u) + Target Area 2 Chr17 Total _____ (from box 7u) = Total Chr17 count _____ _____

8d. Final Result from 40 nuclei: ☐ Non- amplified: HER2/Chr17 < 2.0 or ☐ Amplified: HER2/Chr ≥ 2.0

Scored by: _____ Date: _____