VENTANA®

# INFORM HER2 Dual ISH DNA Probe Cocktail

REF 800-4422

05899826001

## Table of Contents

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## INTENDED USE

The INFORM HER2 Dual ISH DNA Probe Cocktail is designed to quantitatively detect amplification by light microscopy of the HER2 gene via two color chromogenic in situ hybridization (ISH) in formalin-fixed, paraffin-embedded tissue specimens of human breast cancer and gastric cancer, including the gastroesophageal junction, following staining on Ventana automated slide stainers.

The INFORM HER2 Dual ISH DNA Probe Cocktail is indicated as an aid in the assessment of patients for whom Herceptin (trastuzumab) treatment is being considered. This product should be interpreted by a qualified reader in conjunction with histological examination, relevant clinical information, and proper controls.

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

## SUMMARY AND EXPLANATION

HER2/neu (HER2) is a member of a family of four transmembrane receptor tyrosine kinases that mediate the growth, differentiation, and survival of cells.[1,2] The HER2 gene encodes the HER2 protein,[2,3] and overexpression of the HER2 protein, amplification of the HER2 gene, or both, occur in approximately 15 to 25 percent of breast cancers and are associated with aggressive tumor behavior.[4,5] The INFORM HER2 Dual ISH DNA Probe Cocktail contains a HER2 probe (labeled with the hapten dinitrophenyl or DNP) and a Chromosome 17 probe (labeled with the hapten digoxigenin or DIG) formulated with human placental blocking DNA in a formamide-based buffer. The probes are designed to detect amplification of the HER2 gene in breast and gastric carcinoma. The HER2 DNA Probe spans approximately 200,000 base pairs along the genomic region containing the HER2 gene (also known as ERBB2 and NEU), which is located on human Chromosome 17 (17q11.2-q2) (1). The Chromosome 17 probe spans the alpha-satellite 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/Chr17 ratio ≥2.0 is amplified, while a ratio <2.0 is non-amplified).

In many clinical studies, amplification and/or overexpression of HER2 has been shown to be associated with a poor clinical outcome for women with invasive breast cancer, and correlated with several negative prognostic variables, including estrogen receptor (ER) negative status, high S-phase fraction, positive nodal status, mutated p53, and high nuclear grade.[6,7] In several studies, invasive breast cancer patients (both node positive and node negative) with an amplified HER2 gene status displayed decreased overall survival and a higher frequency of recurrence.[1,8,9,10,11] Results from clinical studies measuring HER2 protein over-expression by immunohistochemistry were similar to those obtained by HER2 gene amplification.[9,11,12,13] Knowledge of HER2 gene and/or protein status in invasive breast cancer patients enables clinicians to make more informed decisions to improve the overall management of care for these patients.

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.[14-19] Demonstration of HER2 gene amplification and/or protein overexpression is essential for selecting patients for trastuzumab therapy. Clinical studies have shown that breast cancer patients with high HER2 protein over-expression and/or gene amplification benefit most from trastuzumab.[20] Determination of HER2 gene amplification and/or protein over-expression is necessary for invasive breast cancer patients for whom trastuzumab therapy is being considered and clinically indicated. A percentage of gastric patients exhibit HER2 gene amplification and may benefit from Herceptin therapy.[27] The INFORM HER2 Dual ISH DNA Probe Cocktail assay recommended staining protocol, troubleshooting guidelines, and scoring algorithm apply to both breast and gastric carcinoma specimens.

## PRINCIPLE OF THE PROCEDURE

The INFORM HER2 Dual ISH DNA Probe Cocktail is optimally formulated for use with Ventana *ultra*View SISH DNP Detection Kit, Ventana *ultra*View Red ISH DIG Detection Kit, and accessory reagents on Ventana automated slide stainers. 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 *ultra*View SISH DNP Detection Kit, which contains the following dispensers: a Rabbit anti-DNP Monoclonal Antibody, Multimer solution which contains a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP), Silver ISH DNP Chromogen A (Silver A), Silver ISH DNP Chromogen B (Silver B) and Silver ISH DNP Chromogen C (Silver C). Following incubation with primary Rabbit anti-DNP Antibody and then goat anti-rabbit HRP secondary antibody conjugate, the SISH reaction occurs. Briefly described, this reaction is driven by the sequential addition of Silver A (silver acetate), Silver B (hydroquinone) and Silver 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 (Silver C). The silver precipitate is deposited in the nuclei and a single copy of the HER2 gene is visualized as a black signal. Figure 1 illustrates the SISH reaction.

Following SISH detection for HER2, the DIG-labeled Chromosome 17 probe is detected with the *ultra*View Red ISH DIG Detection Kit. This kit includes the following dispensers: a mouse anti-DIG monoclonal antibody, Red ISH Multimer solution which contains a goat anti-mouse IgG antibody conjugated to Alkaline Phosphatase (AP), pH Enhancer, Naphthol, and Fast Red. Following development of the SISH signal, the slide is incubated with the mouse anti-DIG antibody, which binds to the DIG hapten on the Chromosome 17 probe. The anti-hapten primary antibody is detected with the Multimer solution (goat anti-mouse IgG conjugated to AP enzyme). The slide is incubated with the pH Enhancer solution which provides the proper salt components/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 2 illustrates the Red ISH reaction. The specimen is then counterstained with Hematoxylin II for interpretation by light microscopy.

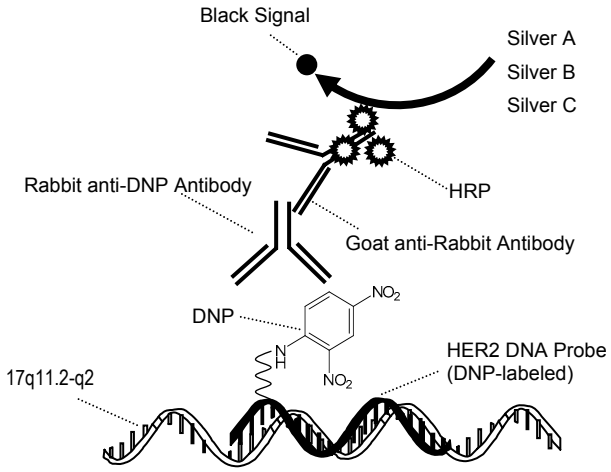



*Figure 1. SISH Reaction for HER2 Detection*

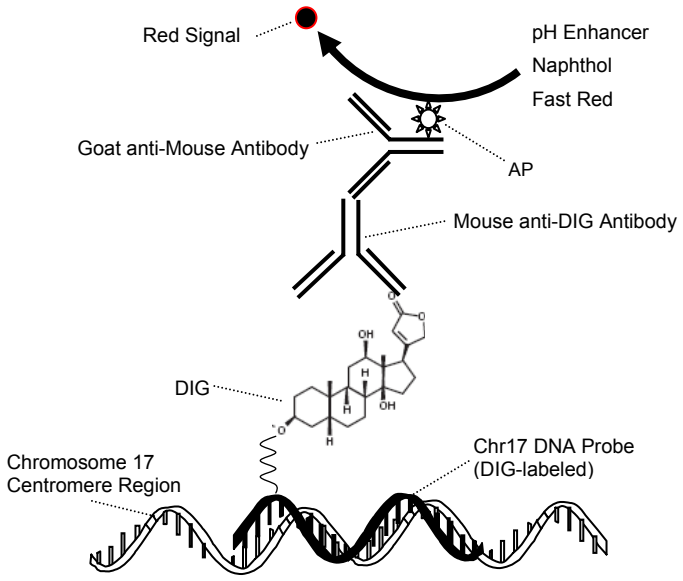



*Figure 2. Red ISH Reaction for Chromosome 17 Detection*

Each step in the automated staining protocol includes incubation of the slide with the specific reagent required for a precise time and temperature. At the end of each incubation step, the sections are rinsed by the Ventana automated slide stainer to stop the reaction and remove unbound material. To minimize evaporation of aqueous reagents from the slide, a coverslip solution is applied by the slide stainer at each step. For more detailed information on instrument operation, refer to the appropriate Ventana automated slide stainer Operator's Manual.

## MATERIALS

### Reagents Provided

INFORM HER2 Dual ISH DNA Probe Cocktail dispenser contains reagent sufficient for 50 tests.

1 - 10 mL dispenser of INFORM HER2 Dual ISH DNA Probe Cocktail contains approximately 12 µg/mL of the HER2 probe labeled with dinitrophenyl (DNP) and 1 µg/mL of the Chromosome 17 probe labeled with digoxigenin (DIG) formulated with human placental blocking DNA in a formamide based hybridization buffer. Both probes are used to determine HER2 gene status (i.e., ratio of HER2 / Chromosome 17).

### Reconstitution, Mixing, Dilution, Titration

No reconstitution, mixing, dilution, or titration is required. Further dilution may result in loss of staining sensitivity. The user must validate any such changes.

### Materials and Reagents Needed But Not Provided

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

1. Ventana *ultra*View SISH DNP Detection Kit [REF 800-098]
2. Ventana *ultra*View Red ISH DIG Detection Kit [REF 800-505]
3. Ventana HybReady Solution [REF 780-4409]
4. Ventana *ultra*View Silver Wash II [REF 780-003 or equivalent]
5. Ventana ISH Protease 2 [REF 780-4148]*
6. Ventana ISH Protease 3 [REF 780-4149]*
7. Ventana Hematoxylin II Counterstain [REF 790-2208]*
8. Ventana Bluing Reagent [REF 760-2037]*
9. Ventana Reaction Buffer (10X) [REF 950-300]
10. Ventana SSC (10X) [REF 950-110]
11. Ventana EZ Prep Reagent (10X) [REF 950-102]
12. Ventana Cell Conditioning 2 (Pre-dilute) [REF 950-123]
13. Ventana ULTRA CC2 (Pre-dilute) [REF 950-223]
14. Ventana Liquid Coverslip (High Temperature) Reagent [REF 650-010]
15. Ventana ULTRA LCS (Pre-dilute) [REF 650-210]
16. Ventana BenchMark Series automated slide stainer
17. Barcode labels
18. Superfrost Plus microscope slides (VWR REF 48311-703 or equivalent)
19. Xylene (Histological grade)
20. Deionized or distilled water
21. Permanent Mounting medium (Permount Fisher Cat. No. SP15-500 or equivalent)**
22. Cover glass (sufficient to cover tissue such as VWR Cat. No. 48393-60 or equivalent)
23. Automated coverslipper (such as Tissue-Tek SCA automated coverslipper)
24. Staining jars or baths
25. Timer
26. Light microscope
27. Ventana HER2 Dual ISH 3-in-1 Xenograft Slides [REF 783-4422] can be used for troubleshooting activities, as needed.

*As needed for specific applications.

**See Table 9 for compatible mounting media with this assay.

### Storage and Handling

Store at 2-8°C. Do not freeze.

To ensure proper reagent delivery and stability of the reagent, after every run replace all dispenser caps and immediately place the dispenser in the refrigerator in an upright position.

Every 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 for the prescribed storage method.

### Specimen Collection and Preparation for Analysis

Routinely processed, formalin-fixed, paraffin-embedded tissues are suitable for use with this reagent. Each section should be cut to the appropriate thickness (approximately 4 µm) and placed on a Superfrost Plus glass slide. The recommended tissue fixative is 10% neutral buffered formalin.[21] Ventana has determined that specimens fixed in zinc formalin or alcoholic formalin also are suitable specimen types. Specimens fixed in Prefer also exhibit single copy detection with this assay, yet the tissue morphology may be affected. It is not recommended that tissues fixed with AFA or Bouin's fixative be used with this assay. Specimens fixed >6 hours with AFA or Bouin's fixatives result in weak or absent staining.[22] Aside from the Ventana assays, recent studies have found that the majority of inconclusive HER2 gene results by FISH relate to pre-analytic factors including under- and over-fixation,[23] as well as delayed fixation.[24] Strict implementation of fixation procedures (e.g., a dedicated processor to ensure a minimum of 6 hours fixation) resulted in a 64% 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.

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 septum 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. These specimens may need to be deparaffinized in alcohol and xylene baths prior to repeat staining on the instrument, or the user may select the extended deparaffinization option in the staining procedure (see Troubleshooting). 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 INFORM 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. Ventana recommends that each laboratory perform initial runs on representative biopsy 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 by Ventana may never stain appropriately with the assay.

## WARNINGS AND PRECAUTIONS

1. For *in vitro* diagnostic (IVD) use.
2. Empty the waste container 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. On the BenchMark XT instrument, a run will not start unless the waste container is emptied prior to starting a run.
3. **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.
4. If reagents come in contact with sensitive areas, wash with copious amounts of water. Avoid inhalation of reagents.
5. Materials of human or animal origin should be handled as potentially biohazardous and disposed of with proper precautions.
6. Avoid microbial contamination of reagents as this could produce incorrect results.
7. Consult local or state authorities with regard to recommended method of disposal.

## INSTRUCTIONS FOR USE

### Step by Step Procedure

This reagent has been developed for use on a Ventana automated slide stainer in combination with Ventana detection kits and accessory reagents. The parameters for the automated procedures can be displayed, printed and edited according to the procedure in the Operator's Manual. Other operating parameters for the automated slide stainers have been preset at the factory. The recommended staining protocol for each instrument platform is listed in Table 1.

*Table 1. Recommended Staining Protocol for INFORM HER2 Dual ISH DNA Probe Cocktail on Ventana BenchMark Series automated slide stainers.*

| Selectable Procedure Step | Recommended Staining Protocol For BenchMark and BenchMark XT | Recommended Staining Protocol For BenchMark ULTRA |
|---|---|---|
| **Baking Temperature** | | Select 63ºC |
| **Baking Time** | | 20 min |
| **Deparaffinization** | Selected | Select 72ºC |
| **Extended Depar*** | Not Selected | Not Selected |
| **Cell Conditioning** | Selected<br>Cell Conditioning CC2<br>Mild CC2 - 8 min<br>Standard CC2 - 12 min<br>Extended CC2 - 8 min | Selected<br>Cell Conditioning CC2<br>86 ºC<br>Mild CC2 - 8 min<br>Standard CC2 - 12 min<br>Extended CC2 - 8 min |
| **ISH-Protease 3** | 16 min (Tissue)<br>8 min (Xenografts) | 16 min (Tissue)<br>8 min (Xenografts) |
| **Denaturation** | 20 min | 20 min |
| **Hybridization** | 6 hours | 6 hours |
| **Stringency wash** | 72°C (Human Tissue)<br>76°C (Xenografts) | 72°C (Human Tissue)<br>76°C (Xenografts) |
| **SISH Multimer** | 16 min | 32 min |
| **Silver Chromogen** | 4 min | 4 min |
| **Red ISH Multimer** | 24 min | 24 min |
| **Red Chromogen** | 8 min | 8 min |
| **Counterstain** | Hematoxylin II - 8 min | Hematoxylin II - 8 min |
| **Post Counterstain** | Bluing Reagent - 4 min | Bluing Reagent - 4 min |

* The Extended Depar option is intended to mitigate severe nuclear bubbling due to excess paraffin.

**Starting a Run on the BenchMark Series Automated Slide Stainers**

1. Apply slide bar code label that corresponds to the probe protocol to be performed.
2. Load the INFORM HER2 Dual ISH DNA Probe Cocktail, reagents from *ultra*View Red ISH DIG and *ultra*View SISH DNP Detection Kits, and required accessory reagents into the reagent tray(s). Place reagent tray(s) on automated slide stainer. Check bulk fluids and waste.
3. The reaction buffer bulk bottles must be full.
4. The waste container must be empty prior to the start of the run.
5. Load slides onto the automated slide stainer.
6. Start the staining run.
7. At the completion of the run, remove slides from the automated slide stainer.
8. Proceed with dehydration procedure.

**Dehydration Procedure**

Note: Fast Red chromogen is soluble in alcohol and acetone. Do not use alcohol and acetone baths to dehydrate slides as the red signal will be affected.

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. Ensure slides are completely dry before coverslipping.
4. Transfer slides into xylene bath for approximately 30 seconds.
5. Place coverslip on slide. Note that some mounting media are not compatible with the assay (See Limitations and Troubleshooting sections).

## Quality Control Procedures

### Specimen Controls

HER2 and Chromosome 17 sequences are present in every cell of the human body. Thus, they act as internal positive controls in every tissue specimen and must be visible (1 to 2 signals per cell) in normal (non-neoplastic) cells in and around the target carcinoma area. However, not all cells will exhibit single gene copy due to biological heterogeneity and truncation from tissue sectioning. Specific nuclear staining may be located in various cells including: stromal fibroblasts, endothelial cells, lymphocytes, and other non-neoplastic cells. If the positive control cells fail to demonstrate positive staining, the slide is considered to be inadequate for enumeration and should be repeated (see Troubleshooting section). Because every normal cell contains two copies of the HER2 gene, there is no true negative specimen control.

A laboratory specific positive specimen control can be run with every staining procedure performed, if desired. 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.

HER2 Dual ISH 3-in-1 Xenograft Slides are used for preliminary validation and troubleshooting activities associated with the INFORM HER2 Dual ISH DNA Probe Cocktail. They contain three distinct xenograft sections that are formalin-fixed and embedded in a single paraffin block. The xenograft sections have been selected because they are well characterized in the scientific literature for HER2 protein levels and gene copy numbers.

### Positive Reagent Control

A positive reagent control can be run during assay verification and troubleshooting activities since DNA accessibility may vary with pre-analytical treatment conditions. The INFORM HER2 Dual ISH DNA Probe Cocktail can be used as a positive control for this assay since every normal cell in the human body contains one to two copies of HER2 and Chromosome 17.

A negative reagent control is not required because internal positive controls are sufficient.

### Unexplained Discrepancies

Unexplained discrepancies in controls should be referred to your local support representative immediately.

### Assay Verification

Prior to initial use of a reagent in a diagnostic procedure, the performance of the reagent should be verified by testing it on a series of specimens with known ISH performance characteristics such as Ventana HER2 Dual ISH 3-in-1 Xenograft Slides (refer to the Quality Control Procedures previously outlined in this section of the product insert). These quality control procedures should be repeated for each new lot of reagents, or whenever there is a change in assay parameters.

## Interpretation of Results

A qualified reader experienced in the microscopic interpretation of breast or gastric carcinoma specimens, ISH procedures and the recognition of single and amplified HER2 copies (which may require microscopic examination using objectives as high as 40X to 60X) must evaluate control nuclei before interpreting results.

HER2 gene status should be enumerated only in the invasive carcinoma. In situ carcinoma (ductal and lobular) in breast specimens should not be enumerated. The reader should refer to the matched H&E slide to determine appropriate target areas to enumerate on the Dual ISH stained slide, if necessary.

### Definitions

1. HER2 Gene Status. HER2 Gene status is a function of the ratio of the average number of copies of the HER2 gene to the average number of copies of Chromosome 17 (Chr17), per cell, in an invasive breast or gastric carcinoma. HER2 gene status is classified using the following guidelines:

   HER2/Chromosome 17 ratio ≥2.0 is amplified

   HER2/Chromosome 17 ratio <2.0 is non-amplified.

2. Slide Adequacy. An individual slide stained with the INFORM HER2 Dual ISH DNA Probe Cocktail must satisfy two criteria to be deemed adequate for enumeration. If the slide does not meet these criteria, then it is inadequate for enumeration.
   a. Internal Positive Control. Normal HER2 (SISH) and Chr17 (Red ISH) signals (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 other non-neoplastic cells. Not every cell on the slide will stain for both probes, but normal cells in and/or around the target must be stained appropriately.
   b. Carcinoma cells. Using 20X, 40X, or 60x objectives, the carcinoma target area must exhibit an enumerable field of SISH and Red ISH signals.
3. Target Areas for Signal Enumeration. An acceptable target area within the carcinoma exhibits an enumerable field of SISH and Red ISH signals. Signal enumeration should not be performed in areas that contain weak SISH and/or Red ISH signals, compressed or overlapping nuclei, or necrosis. Nuclei with excessive SISH or Red ISH background that interferes with the ability to enumerate specific signals should not be counted.

### Additional Observations for HER2 and Chr17

Other observations regarding the HER2 SISH and/or Red ISH Chr17 staining 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 chromosome(s) 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.[25] In cases with clusters of HER2 and Chr17, care must be taken not to consider them with a ratio of ~1.0. The reader should refer to IHC 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 and Slide Adequacy

SISH and Red ISH signals are visualized as:

1. Single Copy. A discrete black (SISH) or red signal (Red ISH) is counted as a single copy of HER2 or Chr17, respectively. For the SISH (black), discrete single signals visualized in the internal, physiologic, same slide positive control (non-neoplastic) nuclei represent the size of a single copy in invasive carcinoma cells. For Red ISH, each discrete signal is counted as one copy. It should be noted that the Red ISH signal from the Chromosome 17-DIG probe may appear larger than the SISH signals, sometimes elongated in shape, and may vary in size within a target area and across samples. SISH signals may appear significantly smaller than Red ISH signals. However, no minimum signal size is required and all discrete signals should be counted. Red 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.
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, each discrete signal is counted as one copy.
3. Clusters. 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 to the extent that signal interpretation is compromised should not be counted.

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

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).

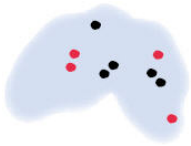
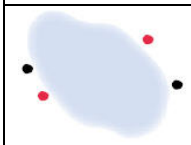
Ventana has developed a scoring algorithm for the assay that maximizes precision and efficiency in counting. Twenty nuclei, each containing red (Red ISH) and black (SISH) signals, should be enumerated. The final results for the HER2 status are reported based on the ratio formed by dividing the sum of 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 (inclusive), 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.

**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

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. Note that heterogeneity is present on the score sheet.

| | |
|---|---|
|  | 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 or greater than the diameter of a single signal. |

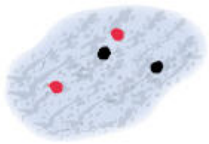
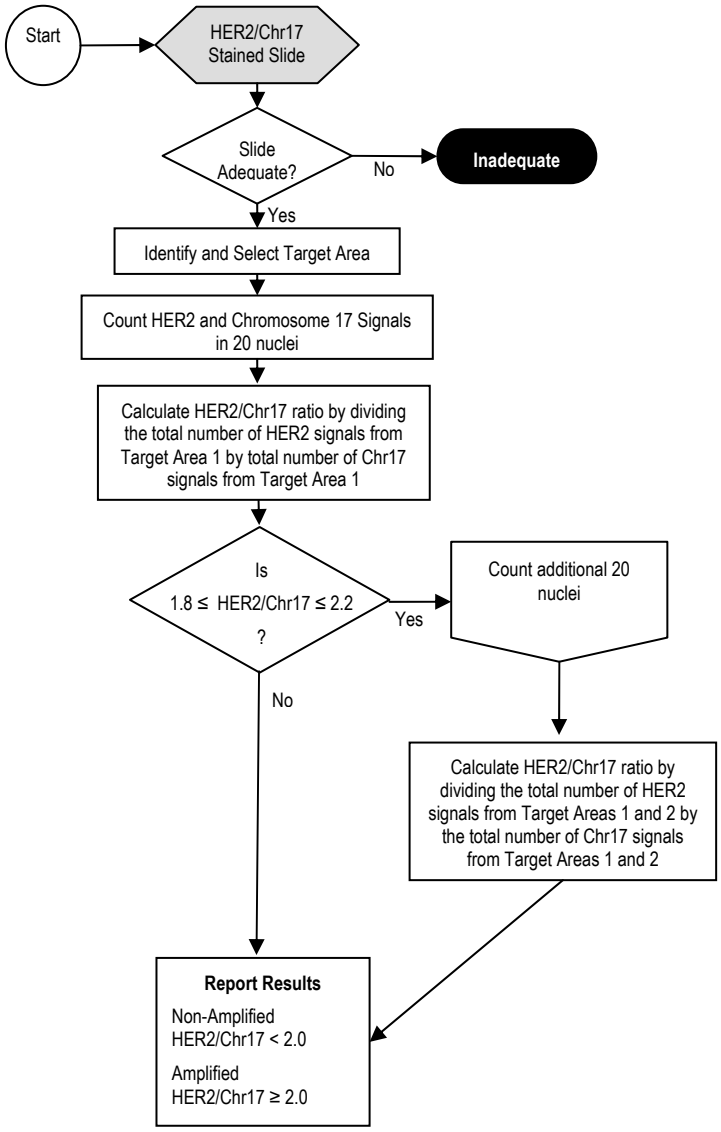
| | |
|---|---|
|  | Small SISH clusters can only be estimated by using the size of a single dot as reference, use stromal cells to estimate signal size (smaller cell on left). For instance, this cluster could be estimated as 6 SISH signals - adding the other 2 single signals yields a total count of 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 Chromosome 17. **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 signals and 2 red signals. If overlapping signals cannot be distinguished, do not count that nucleus. |
|  | Cluster of black signals 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. |
|  | Red haze may be observed and should not be mistaken for signal. Red signal may vary in intensity but is always discrete. The image shows 2 discrete red (Chr17) signals and 2 black (HER2) signals. |

*Table 2.* Signal Visualization for the INFORM HER2 Dual ISH DNA Probe Cocktail.

**HER2 Gene Status: Scoring Algorithm for the 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/Chromosome 17 ratio ≥2.0 and as Non-Amplified if the HER2/Chromosome 17 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**

The presence of silver and red deposition within normal cell nuclei is indicative of positive reactivity. Normal cell nuclei should contain an average of 1-2 discrete SISH and Red ISH signals, indicating that the HER2 DNA and centromeric Chromosome 17 probes have hybridized to the gene and its chromosome, respectively. Failure to detect single gene copy in normal cells indicates slide inadequacy and the results should be considered invalid.

The patient's morphologic findings and pertinent clinical data must be interpreted by a qualified pathologist.

## 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. 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 any positive staining, or its absence, 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 provides reagents at optimal dilution for use when the provided instructions are followed. Further dilution may result in loss of appropriate staining; the user must validate any such change. 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 increase or decrease protease incubation time, cell conditioning time, or the incubation times of the detection components for optimal staining. Such changes must be validated by the user. Users who deviate from recommended test procedures must accept responsibility 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. The INFORM HER2 Dual ISH DNA Probe Cocktail was developed to stain tissue sections that are fixed in 10% NBF and cut at ~4 µm in thickness.[26] The assay also stains specimens fixed in alcoholic formalin, zinc formalin, and Prefer. Fixation in Bouin's or AFA is not recommended.
2. 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.
3. The SISH signal is known to fade after exposure to certain mounting media. See Table 9 in the Troubleshooting section for a list of incompatible mounting media.

## PERFORMANCE CHARACTERISTICS

1. Analytical sensitivity of the INFORM HER2 Dual ISH DNA Probe Cocktail was evaluated using HER2 Dual ISH 3-in-1 Xenograft Slides that were previously characterized for HER2 gene copy number by Abbott/Vysis PathVysion HER-2 DNA Probe Kit for fluorescent in situ hybridization (FISH) and determined to have similar HER2/Chr17 ratios. In addition, ~90 human specimens (a mix of amplified and non-amplified cases) were stained using INFORM HER2 Dual ISH DNA Probe Cocktail and enumerated by two qualified readers. The same cohort was stained using PathVysion FISH and read by one qualified reader. For the INFORM HER2 Dual ISH DNA Probe Cocktail assay, the first pass staining success rate was >93%. The results detailing negative, positive and overall agreement rates for the approximately 60 clinical samples of this cohort that were enumerable with both FISH and the INFORM HER2 Dual ISH DNA Probe Cocktail are shown in Table 3 and Table 4.

*Table 3. Agreement between INFORM HER2 Dual ISH DNA Probe Cocktail and FISH in a cohort of human breast carcinoma specimens across 2 readers.*

| | | FISH Amplification Status | |
|---|---|---|---|
| HER2 Dual ISH Reader | HER2 Dual ISH Amplification Status | Amp | Non-Amp |
| Reader A | Amp | 21 | 2 |
| | Non-Amp | 1 | 40 |
| Reader B | Amp | 17 | 1 |
| | Non-Amp | 2 | 41 |

*Table 4. Summary of negative, positive, and overall agreement rates for INFORM HER2 Dual ISH DNA Probe Cocktail and FISH on human breast carcinoma specimens.*

| HER2 Dual ISH Reader | 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) |
| Reader A | 40/42 | 95.2 (84.2 - 98.7) | 21/22 | 95.5 (78.2 - 99.2) | 61/64 | 95.3 (87.1 - 98.4) |
| Reader B | 41/42 | 97.6 (87.7 - 99.6) | 17/19 | 89.5 (68.6 - 97.1) | 58/61 | 95.1 (86.5 - 98.3) |

2. Analytical specificity (hybridization efficacy) of the INFORM HER2 Dual ISH DNA Probe Cocktail was determined by staining normal human metaphase spreads with the HER2 Dual ISH DNA Probe Cocktail on a BenchMark XT instrument. Of 100 metaphase spreads analyzed, 100% exhibited specific co-localization of both HER2 and Chromosome 17 probes.
3. Reproducibility of the INFORM HER2 Dual ISH DNA Probe Cocktail assay was tested across five different human breast carcinoma cases (representing the dynamic range of HER2 gene status) and HER2 Dual ISH 3-in-1 Xenograft Slides over five non-consecutive days on six instruments (2 BenchMark, 2 BenchMark XT, 2 BenchMark ULTRA). The mean HER2 and Chromosome 17 copy numbers were obtained from each instrument (across the 3 platforms) on each of the five runs. Greater than 98% of all slides stained and evaluated in this study (360 total) passed slide adequacy and resulted in reproducible HER2 and Chromosome 17 copy numbers with %CVs < 10% across all days, instruments and platforms tested.
4. Lot to lot reproducibility of the INFORM HER2 Dual ISH DNA Probe Cocktail assay was determined by testing each of 3 lots of the INFORM HER2 Dual ISH DNA Probe Cocktail with 3 lots of *ultra*View SISH DNP and *ultra*View Red ISH DIG Detection Kits on duplicate slides of 3 human breast carcinoma cases and HER2 Dual ISH 3-in-1 Xenograft Slides. All slides (100%) passed slide adequacy and were enumerated by one qualified reader for raw HER2 and Chr17 copies in 20 nuclei/specimen. The data were subjected to a variance component analysis based on a random effects model, and the results show that all acceptance criteria were met in this study. The %CVs across probe lot, detection kit lot, and within run all were <11%, indicating excellent precision of the assay.
5. Comparability of the assay on breast specimens was determined by a study comparing the INFORM HER2 DNA Probe assay where HER2 and Chromosome 17 copy numbers were determined on individual slides using single SISH detection to the INFORM HER2 Dual ISH DNA Probe Cocktail assay where HER2 and Chromosome 17 copy numbers were determined on a single slide using SISH and Red ISH detection. A cohort of 213 breast carcinoma cases containing ~50/50 mix of non-amp and amplified gene status was tested with both assays on BenchMark XT automated staining instruments (Table 5). The overall agreement rate in clinical samples of the INFORM HER2 Dual ISH DNA Probe Cocktail with INFORM HER2 DNA Probe assay is shown in Table 6.
6. Reproducibility of the INFORM HER2 Dual ISH DNA Probe Cocktail assay was tested across four different human gastric carcinoma cases (representing the dynamic range of HER2 gene status) over five non-consecutive days on six instruments (2 BenchMark, 2 BenchMark XT, 2 BenchMark ULTRA). The mean HER2 and Chromosome 17 copy numbers were obtained from each instrument (across the 3 platforms) on each of the five runs. Greater than 98% of all slides

stained and evaluated in this study (240 total) passed slide adequacy and resulted in reproducible HER2 and Chromosome 17 copy numbers with %CVs < 10% across all days, instruments and platforms tested.

7. Comparability of the assay on gastric specimens was determined by comparing the staining results from the INFORM HER2 Dual ISH DNA Probe Cocktail assay run on a BenchMark XT with the Dako HER2 FISH PharmDx Kit (Table 7). The overall agreement rate in clinical samples of the INFORM HER2 Dual ISH DNA Probe Cocktail with the Dako HER2 FISH PharmDx Kit is shown in Table 8.

*Table 5. Agreement between INFORM HER2 Dual ISH DNA Probe Cocktail using Dual SISH and Red ISH Detection and INFORM HER2 DNA Probe using single SISH detection, on a cohort of invasive breast carcinoma specimens.*

| | HER2 SISH Amplification Status | | |
|---|---|---|---|
| HER2 Dual ISH Amplification Status | Amplified | Non-Amplified | Total |
| Amplified | 101 | 7 | 108 |
| Non-Amplified | 13 | 92 | 105 |
| Total | 114 | 99 | 213 |

*Table 6. Summary of the overall agreement rate for INFORM HER2 Dual ISH DNA Probe Cocktail using Dual SISH and Red ISH Detection compared to INFORM HER2 DNA Probe using single SISH detection on invasive breast carcinoma specimens. The 95% confidence intervals also are presented.*

| Overall Agreement Rate | |
|---|---|
| Raw Data /# of Cases | Percent (95% Score CI) |
| 193/213 | 90.6 (85.9 - 93.8) |

*Table 7. Agreement between the INFORM HER2 Dual ISH DNA Probe Cocktail assay and the Dako PharmxDx FISH test in gastric carcinoma specimens.*

| | Dako FISH Amplification Status | | |
|---|---|---|---|
| HER2 Dual ISH Amplification Status | Amplified | Non-Amplified | Total |
| Amplified | 15 | 2 | 17 |
| Non-Amplified | 6 | 123 | 129 |
| Total | 21 | 125 | 146 |

*Table 8. Summary of the overall agreement rate between the INFORM HER2 Dual ISH DNA Probe Cocktail assay and Dako FISH for HER2 gene status on gastric carcinoma specimens. The 95% confidence intervals also are presented.*

| Overall Agreement Rate | |
|---|---|
| Raw Data /# of Cases | Percent (95% Score CI) |
| 138/146 | 94.5 (89.6-97.2) |

## TROUBLESHOOTING

1. It is critical to evaluate the presence of appropriate signal in the Internal Positive Control nuclei. Normal HER2 and Chr17 signals (1 to 2 copies per cell) act as internal positive controls and their presence confirms assay sensitivity on individual slides. This nuclear staining may be located in various non-neoplastic cells including: stromal fibroblasts, endothelial cells, lymphocytes, and other non-neoplastic cells.
2. Weak or no staining of specimens on a run may indicate a problem with reagents or the instrument. Check that the proper pre-analytical conditions have been followed (See Specimen Collection and Preparation for Analysis). Check to ensure all dispensers are functioning properly. The use of HER2 Dual ISH 3-in-1 Xenograft Slides can be used for troubleshooting if reagent or instrument issues are suspected.
3. If SISH signal is adequate but Red ISH signal is weak or absent, ensure that the slides have not been dehydrated in alcohols or acetone, and have not had prolonged exposure to xylene baths. If Red ISH signal is adequate but SISH is weak or absent (or appears to be fading or turning brown/orange), oxidation of the signal may be occurring. Ensure that the correct mounting media has been used (See Table 9).
4. Specimens that have been improperly collected, fixed, or stored may not exhibit appropriate staining (See Specimen Collection and Preparation for Analysis).
5. If the signal for one or both probes appears weak or absent and the tumor nuclei appear intact and/or blue in color, increasing the pre-treatment condition times is recommended. Specifically, the use of ISH Protease 3 for more than 16 minutes (i.e., 24 minutes) or ISH Protease 2 for 4 minutes or more can be performed. Increased cell conditioning times (16 minutes per cycle for 3 cycles) also is effective. Ventana has determined that manipulation of the pre-treatment steps is most effective at rescuing weak staining.
6. If the signal from the recommended conditions is weak or absent in the tumor cells and the tumor cells appear over-digested (i.e., pale or absent counterstain), this may be due to under-fixation (See Specimen Collection and Preparation for Analysis). Decreasing the ISH Protease 3 time to 8 or 12 minutes is recommended. Additionally, increasing the incubation times of the SISH HRP Multimer, Silver C, AP Multimer, and Fast Red Chromogen also is effective at rescuing weak staining.
7. If the slides demonstrate non-specific SISH background staining ("dust") in the nuclei that may interfere with enumeration of the specific SISH signal, decreasing the Protease 3 time from 16 minutes to 4, 8, or 12 can be performed.
8. If non-specific Red ISH staining in the nucleus interferes with enumeration, re-stain the slide using higher temperatures for the stringency wash (i.e., 76°C).
9. For corrective actions, refer to the Step By Step Procedure section in the automated slide stainer Operator's Manual or contact your local support representative.
10. Sections thicker than 4 µm may require stronger protease conditions to unmask the target DNA. Thinner sections may require gentler protease conditions. In addition, thick sections may exhibit more nuclear bubbling than thinner sections due to excess paraffin in the tissue (See Specimen Collection and Preparation for Analysis). These may need to be deparaffinized in alcohol and xylene baths prior to staining on the instrument, or the user can select the "extended deparaffinization" option in the staining procedure to mitigate nuclear bubbling due to excess paraffin.
11. If a case exhibits uninterpretable staining, with brown, black or dark red background throughout the tissue, the slide must be re-run. If staining is still unacceptable, ensure that the slides are SuperFrost Plus. If excess Silver or Red is deposited on the slide, making it difficult to enumerate the nuclear signal due to speckling throughout the tissue, the slide must also be re-run. Usually, no more than 5-6% of cases should have to be repeated due to these drying/speckling artifacts.

*Table 9. Mounting Media tested for compatibility with SISH based assays.*

| Mounting Media | Manufacturer | Type (Xylene, alcohol, aqueous) | Result (Normal staining = N; Fading = F) |
|---|---|---|---|
| Eukitt | EMS | Xylene | **F** |
| Entellan New | Merck | Xylene | **F** |
| Entellan | Merck | Xylene | **F** |
| HSR | Sysmex | Xylene | **F** |
| Malinol | Muto Chemical | Xylene | **F** |
| Cytoseal XYL | Richard Allan Scientific | Xylene | N |
| Softmount | WAKO | Lemasol A | N |
| Paramount | Protaqs Quartett: Dako | Xylene | N |
| DPX | BDH: Raymond Lamb | Xylene | N |
| Cytoseal 60 | Richard Allan Scientific | Xylene | N |
| Permount | Fisher | Xylene | N |
| Histomount | Raymond Lamb | Xylene | N |
| Ultramount | Dako | Xylene | N |
| Thermo EZ Mount | Thermo Scientific | Xylene | N |
| SureMount | Triangle Biomedical Sciences | Xylene | N |
| Flo-Texx | Lerner Labs | Xylene | N |
| Mountex | Histolab | Xylene | N |
| Shandon Consul mount | Thermo Scientific | Xylene | N |
| MM24 | SurgiPath | Xylene | N |
| Pertex | Cell Path | Xylene | N |
| MicroMount | SurgiPath | Xylene | N |
| Diamount | Diapath | Xylene | N |
| Alcolmount | Diapath | Alcohol | N |
| BioMount 2 | BBInternational | Xylene | N |
| Acrytol | SurgiPath | Xylene | N |
| Gel Mount | Biomeda | Aqueous | N |
| Mount-Quick | Daido Sangyo Co. | Aqueous | N |

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## INTELLECTUAL PROPERTY



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## Appendix A: Interpretation of Results Scoring Sheet

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

| **Target Area 1** | | | |
|---|---|---|---|
| ☐ Heterogeneity present? (Check if yes) | | | |
| Cell | HER2 Count | Cell | Chr17 Count |
| 1 | | 1 | |
| 2 | | 2 | |
| 3 | | 3 | |
| 4 | | 4 | |
| 5 | | 5 | |
| 6 | | 6 | |
| 7 | | 7 | |
| 8 | | 8 | |
| 9 | | 9 | |
| 10 | | 10 | |
| 11 | | 11 | |
| 12 | | 12 | |
| 13 | | 13 | |
| 14 | | 14 | |
| 15 | | 15 | |
| 16 | | 16 | |
| 17 | | 17 | |
| 18 | | 18 | |
| 19 | | 19 | |
| 20 | | 20 | |
| ☐Clusters Present? (Check if yes) | | ☐Clusters Present? (Check if yes) | |
| Total number of HER2 signals in Target Area 1 | | Total number of Chr17 signals in Target Area 1 | |
| a | | b | |
| Target Area 1 HER2/Chr17 Ratio | | | |
| c = a/b | | | |
| ☐ **Non-amplified: HER2/Chr17 < 2.0** | | | |
| ☐ **Amplified: HER2/Chr17 ≥ 2.0** | | | |

| **Target Area 2 If ratio 1.8 ≤ HER2/Chr17 ≤ 2.2** | | | |
|---|---|---|---|
| ☐ Heterogeneity present? (Check if yes) | | | |
| Cell | HER2 Count | Cell | Chr17 Count |
| 1 | | 1 | |
| 2 | | 2 | |
| 3 | | 3 | |
| 4 | | 4 | |
| 5 | | 5 | |
| 6 | | 6 | |
| 7 | | 7 | |
| 8 | | 8 | |
| 9 | | 9 | |
| 10 | | 10 | |
| 11 | | 11 | |
| 12 | | 12 | |
| 13 | | 13 | |
| 14 | | 14 | |
| 15 | | 15 | |
| 16 | | 16 | |
| 17 | | 17 | |
| 18 | | 18 | |
| 19 | | 19 | |
| 20 | | 20 | |
| ☐Clusters Present? (Check if yes) | | ☐Clusters Present? (Check if yes) | |
| Total number of HER2 signals in Target Area 2 | | Total number of Chr17 signals in Target Area 2 | |
| d | | e | |
| Target Areas **1 and 2** HER2/Chr17 Ratio | | | |
| f = (a+d)/(b+e) | | | |
| ☐ **Non-amplified: HER2/Chr17 < 2.0** | | | |
| ☐ **Amplified: HER2/Chr17 ≥ 2.0** | | | |