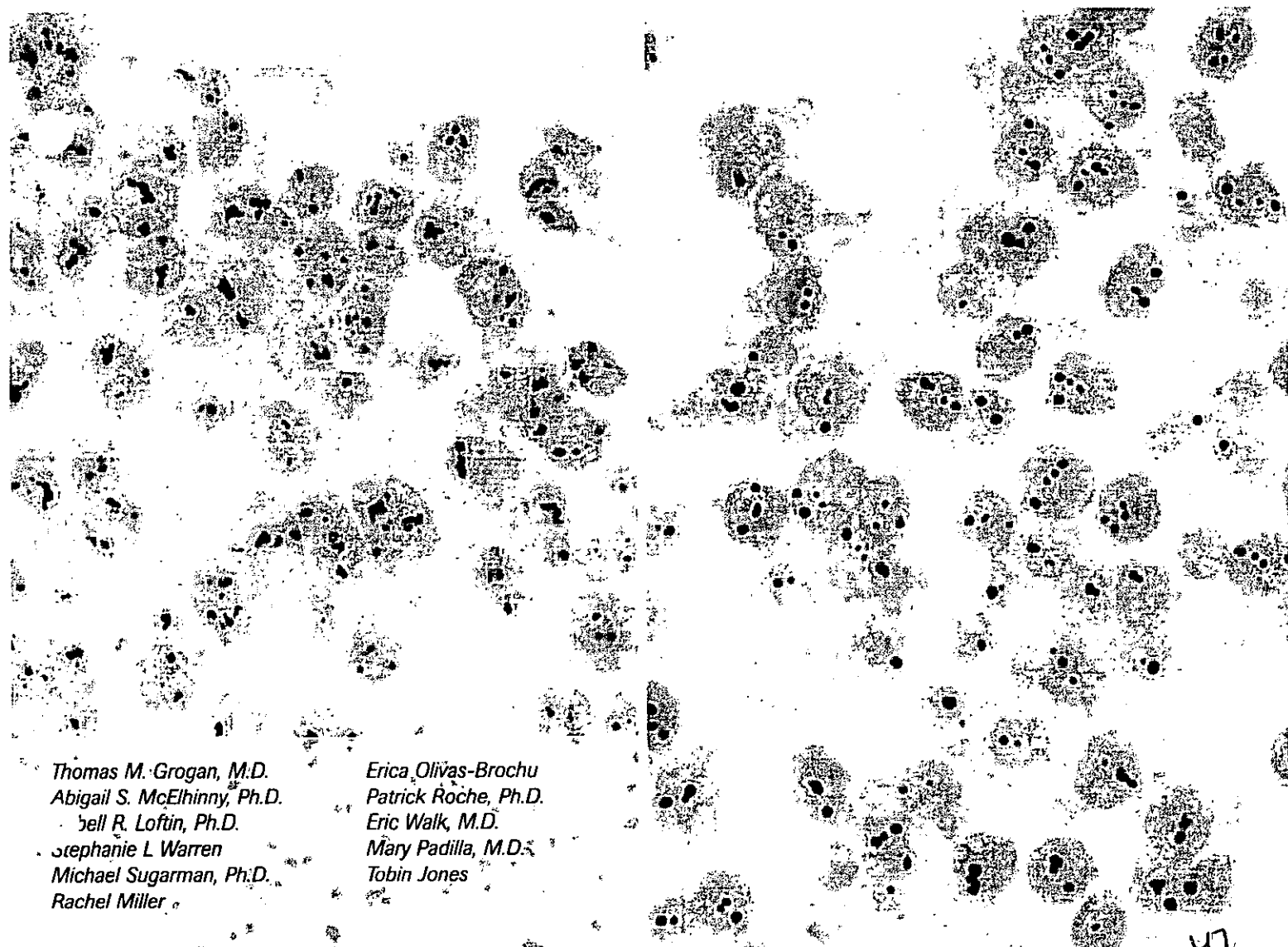


# Interpretation Guide

## *Ventana INFORM HER2 Dual ISH*

### *DNA Probe Cocktail Assay*



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Front cover images:

- HER2 clusters and Chr17 60X, amplified (left)
- HER2 and Chr17 60X, non-amplified (right)

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## Introduction

### General Description of Ventana

#### INFORM HER2 Dual ISH DNA Probe Cocktail assay

Ventana Medical Systems' (Ventana) INFORM HER2 Dual ISH DNA Probe Cocktail is designed to determine HER2 gene status by enumeration of the ratio of the HER2 gene to Chromosome 17. The HER2 and Chromosome 17 probes are detected using two color chromogenic *in situ* hybridization (ISH) in formalin-fixed, paraffin-embedded human breast cancer tissue specimens following staining on Ventana BenchMark XT automated slide stainer (using NexES Software), by light microscopy. The INFORM HER2 Dual ISH DNA Probe Cocktail is indicated as an aid in the assessment of patients for whom Herceptin (trastuzumab) treatment is considered.

The INFORM HER2 Dual ISH DNA Probe Cocktail assay is designed to determine HER2 gene status by detecting: (1) HER2 copies via silver *in situ* hybridization (SISH) and (2) Chromosome 17 (Chr17) copies via chromogenic Red *in situ* hybridization (Red ISH) on a single slide. Before interpreting results, staining of internal positive control nuclei must be evaluated by a qualified reader experienced in the microscopic interpretation of breast 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).

#### Purpose of this Interpretation Guide

This guide is intended to provide pathologists with a tool to facilitate HER2 gene status determination via interpretation of HER2 and Chr17 staining patterns using the INFORM HER2 Dual ISH DNA Probe Cocktail. The following cases illustrate the variety of staining patterns that may be observed in breast specimens following staining with the INFORM HER2 Dual ISH DNA Probe Cocktail (Figures 2-11). These photomicrographs allow a new user to become familiar with the spectrum of staining patterns. These include single copy staining of HER2 and Chr17, multiple copies and clusters of HER2 staining, Chr17 polysomy, as well as artifact staining that may be encountered. Additionally, the images aid in the determination of slide adequacy, enumeration of copy numbers using the scoring algorithm, and assay troubleshooting.

Any staining performed in the end user's laboratory should be interpreted within the context of the internal positive control nuclei that are present in each clinical case. For further information, see the package inserts online ([www.ventanamed.com](http://www.ventanamed.com)), the Quality Control section, and the Slide Adequacy section of this guide.

Images contained in this Interpretation Guide were obtained using the INFORM HER2 Dual ISH DNA Probe Cocktail assay, which was developed and validated on Ventana BenchMark XT, under the direction of Ventana Medical Systems, Inc.

#### What You Can Expect to See Regarding Performance of the HER2 Dual ISH DNA Probe Cocktail in your Laboratory

For the INFORM HER2 Dual ISH DNA Probe Cocktail, the recommendation is that tissue be fixed in 10% neutral buffered formalin (NBF) for six to twenty-four hours, paraffin embedded and sectioned at approximately four microns<sup>1</sup>. Recent studies suggest that the majority of inconclusive HER2 gene results relate to pre-analytic factors including "under" and "over" fixation<sup>2</sup> and delay to fixation<sup>3</sup>. While strict implementation of fixation conditions is possible<sup>2</sup>, it is difficult to precisely control tissue fixation time in reference laboratories receiving samples from multiples sources. To compensate for tissue variations, such as variable pre-analytical factors, this assay has been developed with certain selectable protocol steps, including those within the pre-treatment, hybridization, as well as detection chemistries. These options may enable further optimization of the assay, as needed for specific specimens. Note that a certain percentage of slides (not more than 6%) still may need to be re-stained due to slide drying and/or other artifacts<sup>4</sup>. The slide drying artifact is easy to recognize (see Troubleshooting section for further discussion on selectable protocol options, troubleshooting, and staining artifacts in the assay). In addition, some fixatives are not recommended for ISH-based assays (including Bouin's and AFA). See Pre-analytical consideration section for discussion of fixatives for the INFORM HER2 Dual ISH DNA Probe Cocktail assay.

# Identification of Appropriate Staining Pattern

## HER2 Gene Status

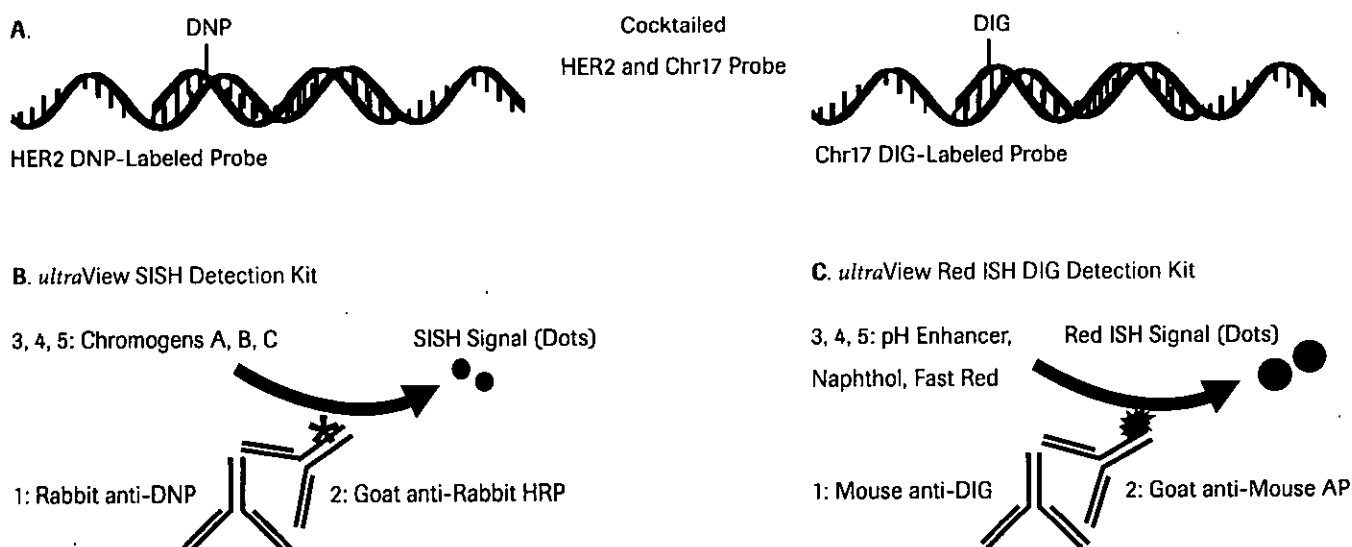
In humans, the HER2 gene, located on Chr17, encodes the HER2 protein. Amplification of the HER2 gene occurs in approximately 15 to 25 percent of breast cancers, and is associated with aggressive tumor behavior.<sup>5-8</sup> In many clinical studies, amplification and/or HER2 overexpression has been 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.<sup>10-11</sup>

Ventana has designed the INFORM HER2 Dual ISH DNA Probe Cocktail to enable the HER2 gene and Chr17 centromere to be co-hybridized and visualized via light microscopy on the same slide. Specifically for this assay, HER2 is detected by a dinitrophenyl (DNP) labeled probe and visualized utilizing *ultraView* SISH DNP (silver *in situ* hybridization) Detection Kit. The Chr17 centromere is targeted with a digoxigenin (DIG) labeled probe and detected using *ultraView* Red ISH DIG Detection Kit. Dual ISH staining results in visualization via light

microscopy in which HER2 appears as discrete black signals (SISH) and Chr17 as red signals (Red ISH) in nuclei of normal cells (serving as internal positive controls for staining) as well as in carcinoma cells. This strategy allows HER2 gene status determination in the context of its chromosomal state, using standard light microscopy with 20X, 40X, and/or 60x objectives.

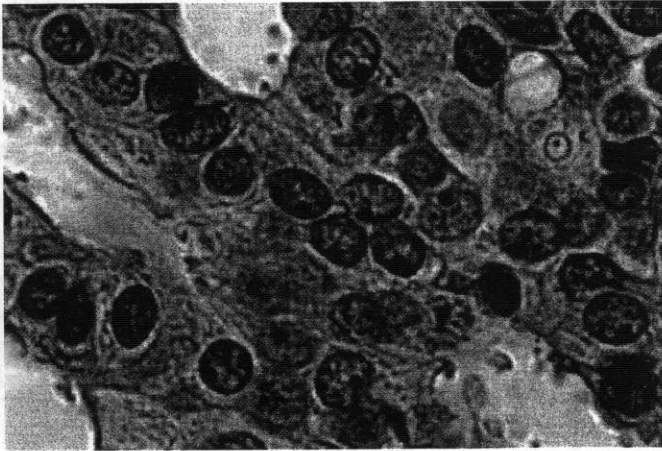
HER2 gene status is reported as a function of the ratio of the average number of HER2 gene copies to the average number of Chr17 copies in nuclei of cells within an invasive breast carcinoma. HER2 gene status is classified as non-amplified (HER2/Chr17 ratio < 2.0: Figure 2, Case 1) or amplified (HER2/Chr17 ratio ≥ 2.0: Figure 2, Case 3). Care must be taken for cases that fall within the 1.8-2.2 ratio range<sup>1</sup>. Ventana has developed a scoring algorithm to determine HER2 gene status following staining with the INFORM HER2 Dual ISH DNA Probe Cocktail. This algorithm is discussed in detail in the Slide Scoring section (Figure 7). Cases representing the dynamic range of HER2 status are shown in Figures 2-4.

Figure 1.

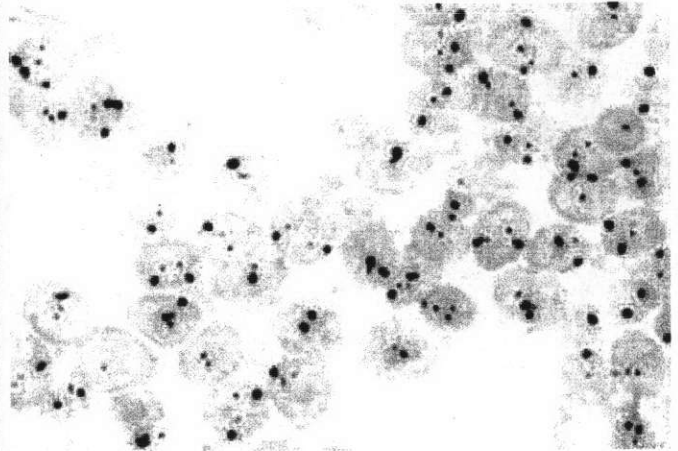


**Figure 2. HER2 gene status representative cases**

**Case 1 is non-amplified for HER2**

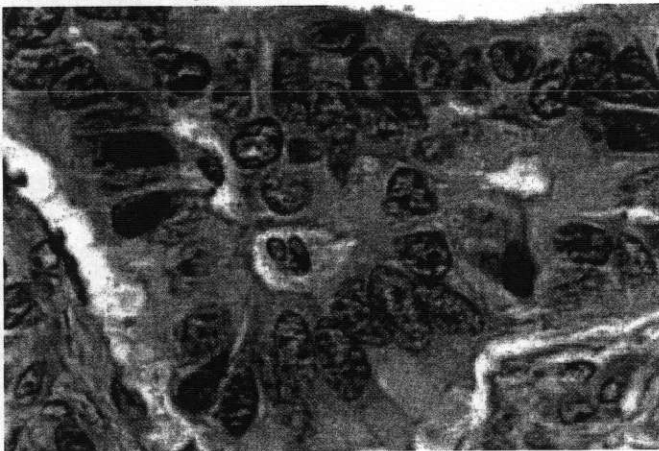


H&E 60X

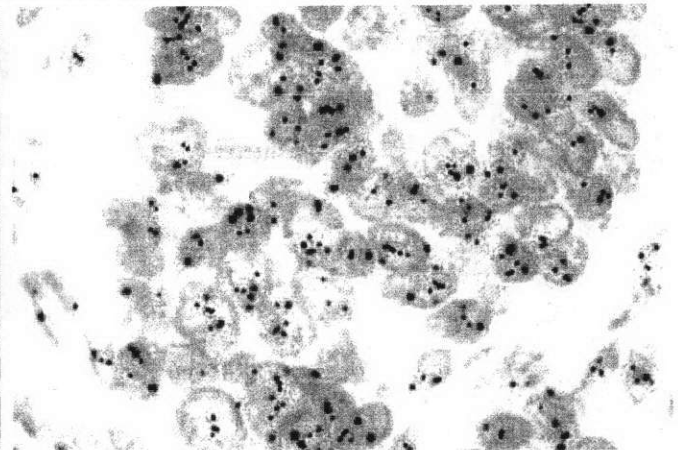


HER2 and Chr17 60X; Distinct SISH an Red ISH Signals are visible as 1-2 copies per tumor cell

**Case 2 is non-amplified for HER2, but contains multiple copies of HER2 and Chr17**



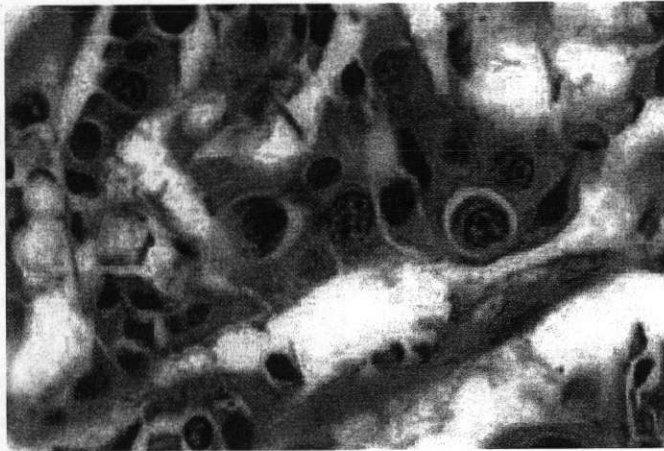
H&E 60X



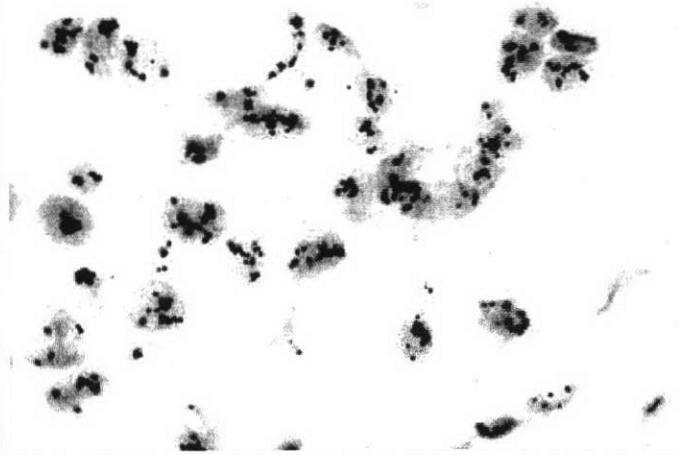
HER2 and Chr17 60X; Greater than 2 SISH Signals are present in the tumor cells, on average.

Figure 2. Continued

Case 3 is HER2 gene amplified with SISH clusters

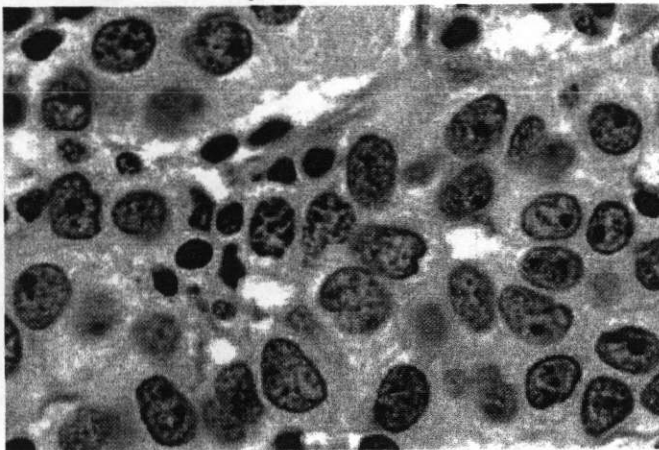


H&E 60X

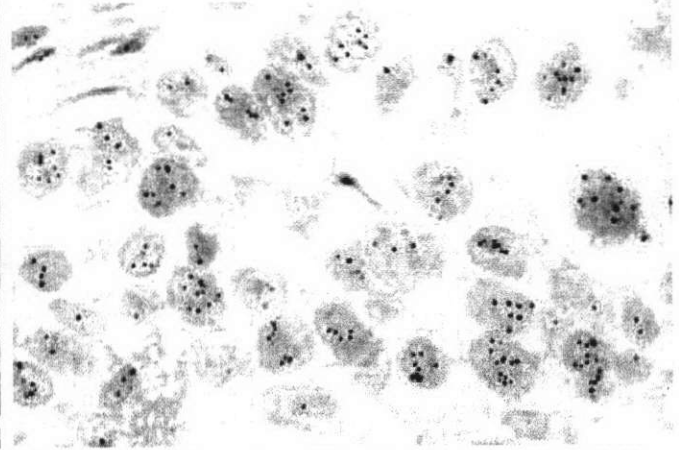


HER2 and Chr17 60X; HER2 SISH clusters are present and are estimated by the reader. 1-2 copies of the Chr 17 Red ISH signal are present

Case 4 is low-level amplified for HER2



H&E 60X



HER2 and Chr17 60X; Greater than 2 HER2 copies are present, but only 1-2 Chr17 copies, on average. This case is low level amplified



## Signal Visualization and Enumeration

ISH signals are visualized as single copies, multiple copies and clusters (Figure 3 and Table 1). Single copies in the normal cells are used as a reference to enumerate the signals in the carcinoma nuclei.

### Single Copy

A discrete signal is counted as a single copy of HER2 or Chr17. Discrete single signals visualized in the internal positive control nuclei represent the size of a single copy in carcinoma cells (Figure 2-4). It is important to note that the discrete signals representing a single copy of either HER2 or Chr17 may appear smaller or larger in some patient samples compared to others. In addition, SISH signals (black) are typically smaller in size and more discrete in appearance than Red ISH signals (red), due to differences in target sizes and detection chemistries. Therefore, it is important to use the single signals visualized in the internal positive control nuclei (the physiologic control) adjacent to the target area (the pathologic lesion) as a reference for relative signal size for SISH signals. Red ISH signals may appear larger than SISH signals, are sometimes elongated in shape, and may vary in size within a target area and across samples. The internal positive control nuclei occur within normal adjacent stromal cells (e.g. fibroblasts/fibrocytes and endothelials) and leukocytes (e.g. lymphocytes and macrophages).

### Multiple Copies

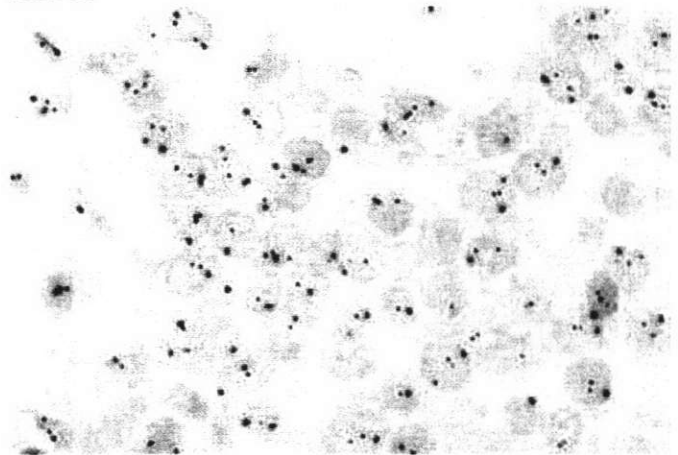
As described above, discrete single black signals (SISH) visualized in the internal positive control nuclei represent single copy size in invasive carcinoma cells. Cases 2, 4, 6 and 9 in Figures 2-4 show a number of nuclei in which multiple discrete copies are visible in the carcinoma nuclei.

### Clusters

A cluster is defined as numerous overlapping SISH signals in the nuclei that cannot be individually discerned. As these are difficult to precisely enumerate, the number of HER2 gene copies in a cluster can only be estimated. For example, a small cluster may be counted as 6 signals, and larger clusters as 12 signals or more. It is possible for a single nucleus to have multiple small clusters, multiple large clusters, a combination of large and small clusters, and/or clusters and single signals. Case 3, 7 and 10 in Figures 2-4 illustrate examples of nuclei in which clusters are visible.

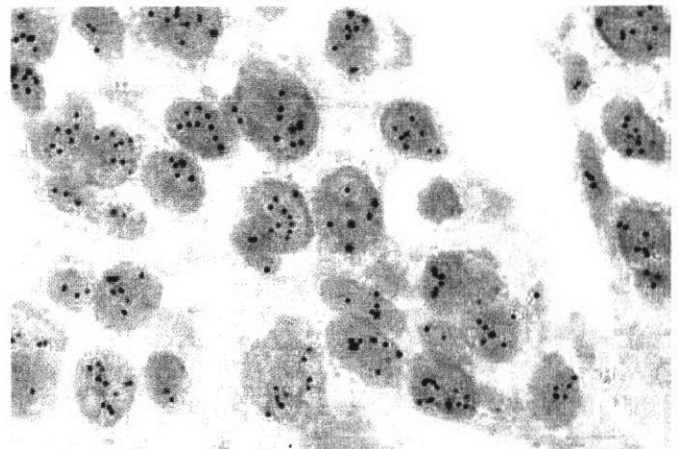
Figure 3. Signal visualization

Case 5.



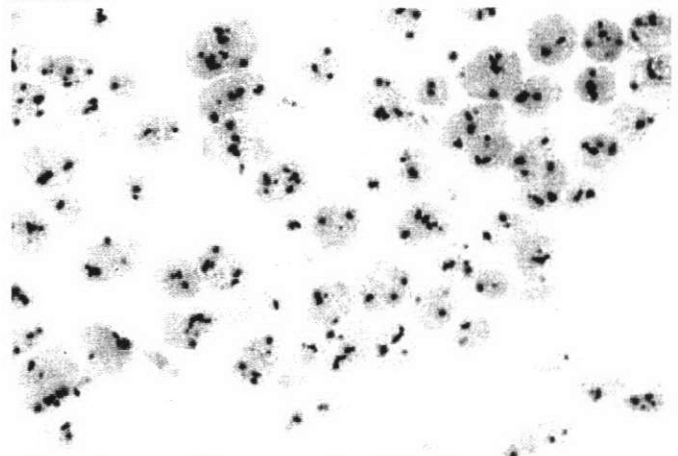
HER2 and Chr17 60X; Single copy HER2, non-amplified HER2, 60X

Case 6.



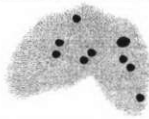

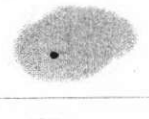
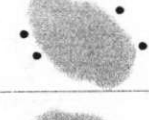

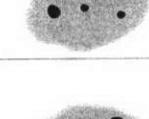
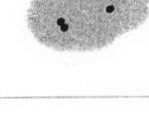

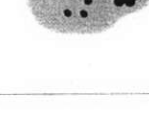

HER2 and Chr17 60X; Multiple single copy HER2, low level amplified HER2, 60X

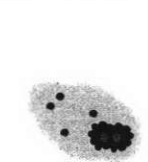
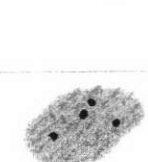
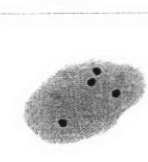
Case 7.



HER2 and Chr17 60X; HER2 clusters, amplified HER2, 60X

**Table 1. 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 signal is 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 are estimated by using the size of a single signal 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 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 signal 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 black (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

**Slide Adequacy must be verified before enumerating the slide**

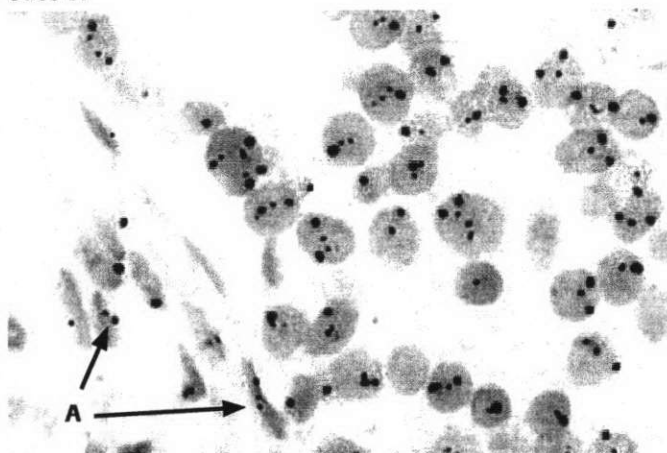
Before enumerating HER2 and Chr17 signals to determine HER2 gene status, it is critical to determine whether the invasive target area (the lesional tissue) is adequately stained and satisfies the criteria described below. If the target is inadequate for enumeration, the user should refer to the Troubleshooting section and evaluate appropriate conditions to follow for repeat staining the slide.

**Criterion 1. Internal positive control staining must be present**

HER2 and Chr17 signals in non-neoplastic nuclei (one to two copies per cell that are visible as distinct "single copy" staining) act as internal, physiologic, "same slide" positive controls and should be visible using 20X, 40X, or 60X objectives. This distinct nuclear staining may be located in the normal cells in and/or around the target area, including: stromal fibroblasts, endothelial cells, lymphocytes and benign breast epithelial cells (See Fig. 3). Due to truncation artifacts in the plane of sectioning, it is usually not possible to visualize single HER2 or Chr17 signals in all cells on the slide, nor in all regions of the tissue. However, accurate enumeration requires that single-copy signals are visible in normal cells within and/or adjacent to the target area.

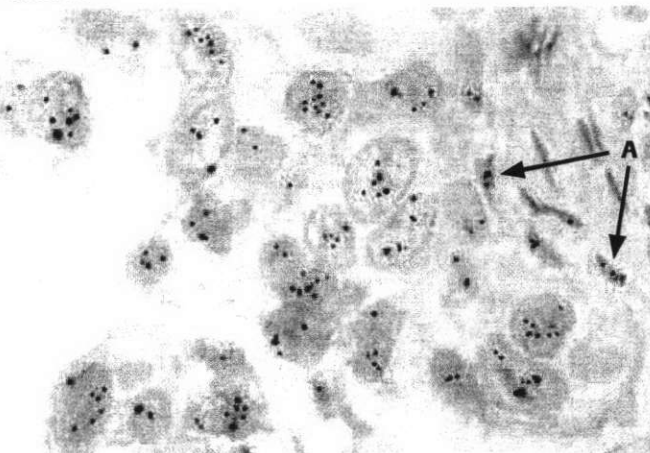
Figure 4. Normal staining in internal positive control nuclei

Case 8.



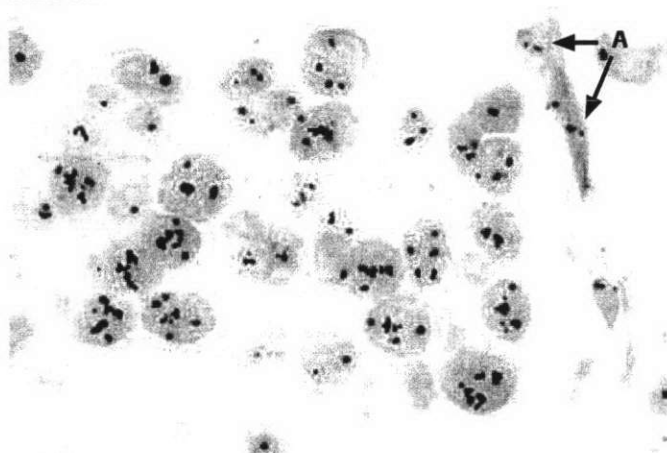
HER2 and Chr17 60X; Non-amplified for HER2  
A: Internal positive control nuclei

Case 9.



HER2 and Chr17 60X; multiple copies of HER2  
A: Internal positive control nuclei

Case 10



HER2 and Chr17 60X; HER2 amplified with SISH clusters  
A: Internal positive control nuclei

Figure 4 contains examples of cases with appropriate internal positive control staining.

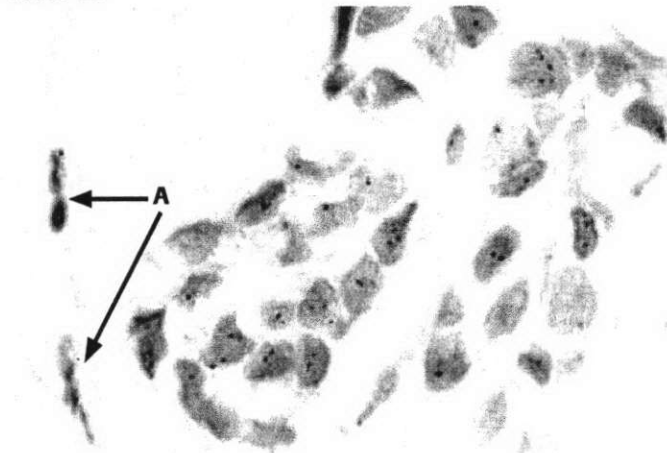
Figure 5 shows examples of cases that are inadequate due to a lack of either SISH or Red ISH staining in both the positive control nuclei and tumor nuclei. Such cases must be repeated before enumeration.

Figure 6 shows a case which is inadequate due to absent staining in the positive control nuclei. Therefore this case cannot be enumerated and must be repeated.

See the Troubleshooting section for repeat staining samples that are inadequate for enumeration.

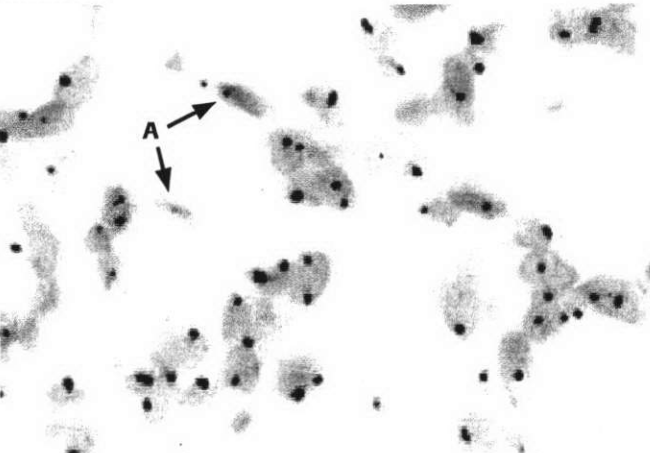
Figure 5. Inadequate due to lack of staining for either SISH or Red ISH

Case 11.



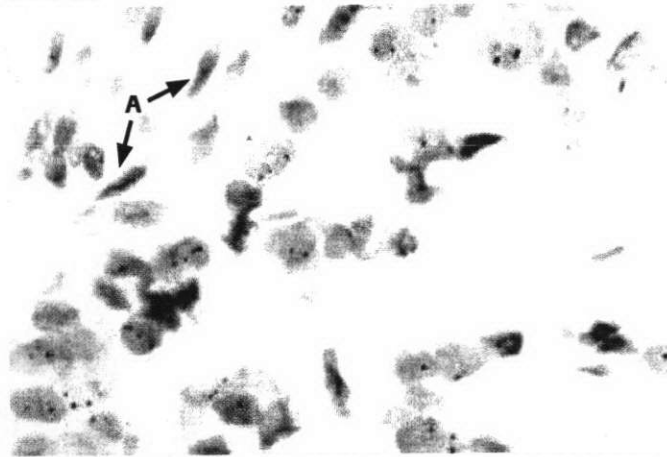
HER2 and Chr17, 60X, inadequate due to weak/absent Red ISH staining in positive control nuclei and tumor nuclei. A: Internal positive control nuclei

Case 12.



HER2 and Chr17, 60X, inadequate due to lack of SISH staining in positive control nuclei and tumor nuclei. A: Internal positive control nuclei

**Figure 6. Inadequate due to lack of staining in positive control nuclei**  
**Case 13.**



60X, inadequate due to lack of staining for both SISH and Red ISH in positive control nuclei. A: Internal positive control nuclei exhibit weak/absent staining for both SISH and Red ISH, although the tumor cells are stained with both probes. This case is inadequate for enumeration

**Criterion 2. Staining within the invasive breast carcinoma cells in the target area must be enumerable**

Using 20X, 40X, and/or 60X objectives, the invasive breast carcinoma in the target area must exhibit an enumerable field of HER2 (SISH) and Chr17 (Red ISH) signals. Due to truncation in the plane of sectioning, it is likely that not every carcinoma nucleus will contain signals. However, it is important that the target area contains an acceptable region that is enumerable. If a particular target area is deemed too weak to enumerate, it is often possible to enumerate a different target area on the same slide. If all larger areas exhibit inadequate staining, then the slide is considered inadequate and cannot be enumerated.

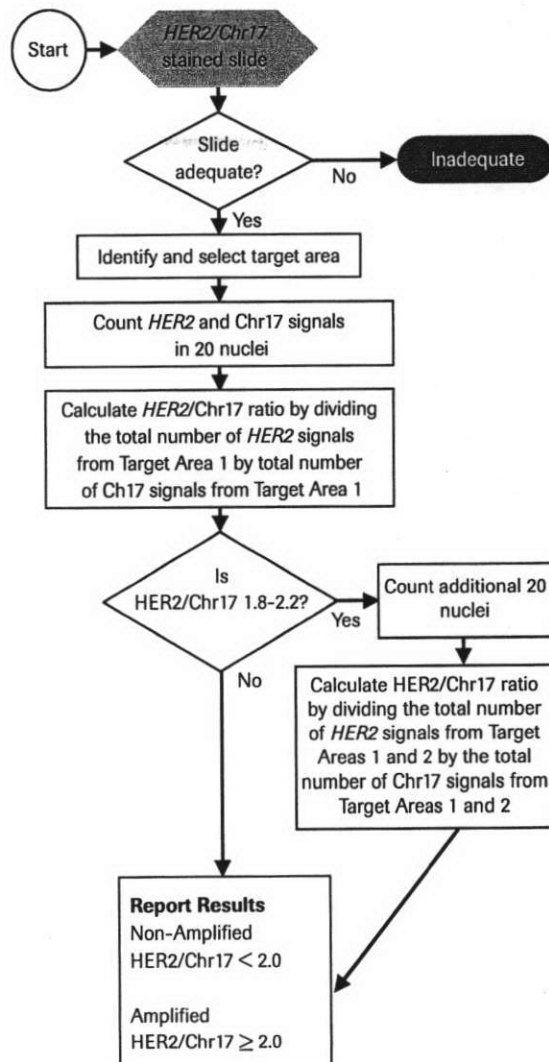
**Criterion 3. Background staining must not interfere with enumeration**

Finally, 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 (Figure 21). Red background may appear as red haze that is fainter in intensity compared to the specific signal. (Figure 20 and 22). See Troubleshooting section for more information on repeat staining cases that may have background staining that interfere with enumeration.

**Slide Scoring**

Ventana has developed a scoring algorithm that maximizes precision and efficiency to determine HER2 gene status. Once an adequate target area is identified, the reader records the scores for HER2 and Chr17 copy numbers that are present in 20 representative nuclei. If the resulting HER2 / Chr17 ratio falls within 1.8-2.2, the reader is recommended to score an additional 20 nuclei and the resulting ratio is calculated from the total 40 nuclei. HER2 gene status is reported as non-amplified (HER2/Chr17 < 2.0) or amplified (HER2/Chr17 > 2.0). A flow diagram of the scoring algorithm is shown below.

**Figure. 7**



## REFERENCE ONLY. Do Not Distribute.

### Summary of scoring

Once a target area that exhibits adequate staining is located, only representative carcinoma cells that contain both SISH (HER2) and Red ISH (Chr17) signals should be enumerated. Signal enumeration should not be performed in areas that contain: weak or absent SISH or Red ISH signals, absent internal control cell staining, compressed or overlapping nuclei, nuclei with excessive Red ISH or SISH background staining or necrosis. Additionally, signal enumeration should not be performed in nuclei that are not representative of the general population of invasive carcinoma nuclei in the target area. For example, abnormally large nuclei (2-fold or greater in size relative to other carcinoma nuclei in the field) and small nuclei (approximately half the size of other carcinoma nuclei) should not be enumerated. Finally, in target areas that are genetically heterogeneous for HER2 copy numbers, count only nuclei that are representative of the population of invasive carcinoma nuclei with the highest average number of signals. Heterogeneity is discussed in greater detail in Additional Observations.

To score a slide stained with the INFORM HER2 Dual ISH DNA Probe Cocktail:

1. Examine H&E stained slide to locate areas containing invasive breast carcinoma
2. Examine HER2 Dual ISH stained slide corresponding to the H&E, and identify an invasive breast carcinoma target area where:
  - The majority of cells in the selected area display hybridization signals for both SISH and Red ISH that are not obscured by non-specific background staining.
  - There are internal positive control cells in each area adjacent to the tumor area to be scored (Note: internal positive control cell staining is not applicable for xenografts, as xenografts are comprised of human

tumor cell lines grown in mice; therefore, the non-tumor cells are mouse cells containing mouse HER2 and Chr17 sequences which will not be detected by either probe. The area is considered adequate for enumeration if one to two copies of HER2 and Chr17 signals are present in various normal cells including: stromal fibroblasts, endothelial cells, lymphocytes and other non-neoplastic cells.)

3. Count 20 representative nuclei within the invasive region per specimen, that meet the following requirements:
  - Compare nuclei not significantly greater or less than median diameter with overall tumor nuclei.
  - Select cells that have minimal or no overlap with other cells. If Red ISH and SISH signals appear overlapping, the reader may have to examine the signals at 60x magnification to discern.
  - Do not count nuclei that are severely over-digested, "bubbled", or are found in areas containing non-specific staining on the slide that could interfere with enumeration. See Troubleshooting for images of these types of nuclei. Also see discussion on Pre-Analytical Factors.
  - Score HER2 and Chr17 signals detectable in 20 representative nuclei.
  - Note the presence of HER2 clusters, and estimate the number of copies based on the size of a single copy. Note that higher number of Red ISH signals visible in nuclei with SISH clusters should be counted.
4. Determine the sum of HER2 and Chr17 copy numbers and calculate the resulting ratio.  
If the ratio falls within 1.8-2.2, an additional 20 nuclei must be counted and the resulting ratio is based on the total of 40 nuclei.