

Interpretation Guide

for VENTANA anti-HER2/neu (4B5)

Rabbit Monoclonal Primary Antibody Staining
of Breast and Gastric Carcinoma

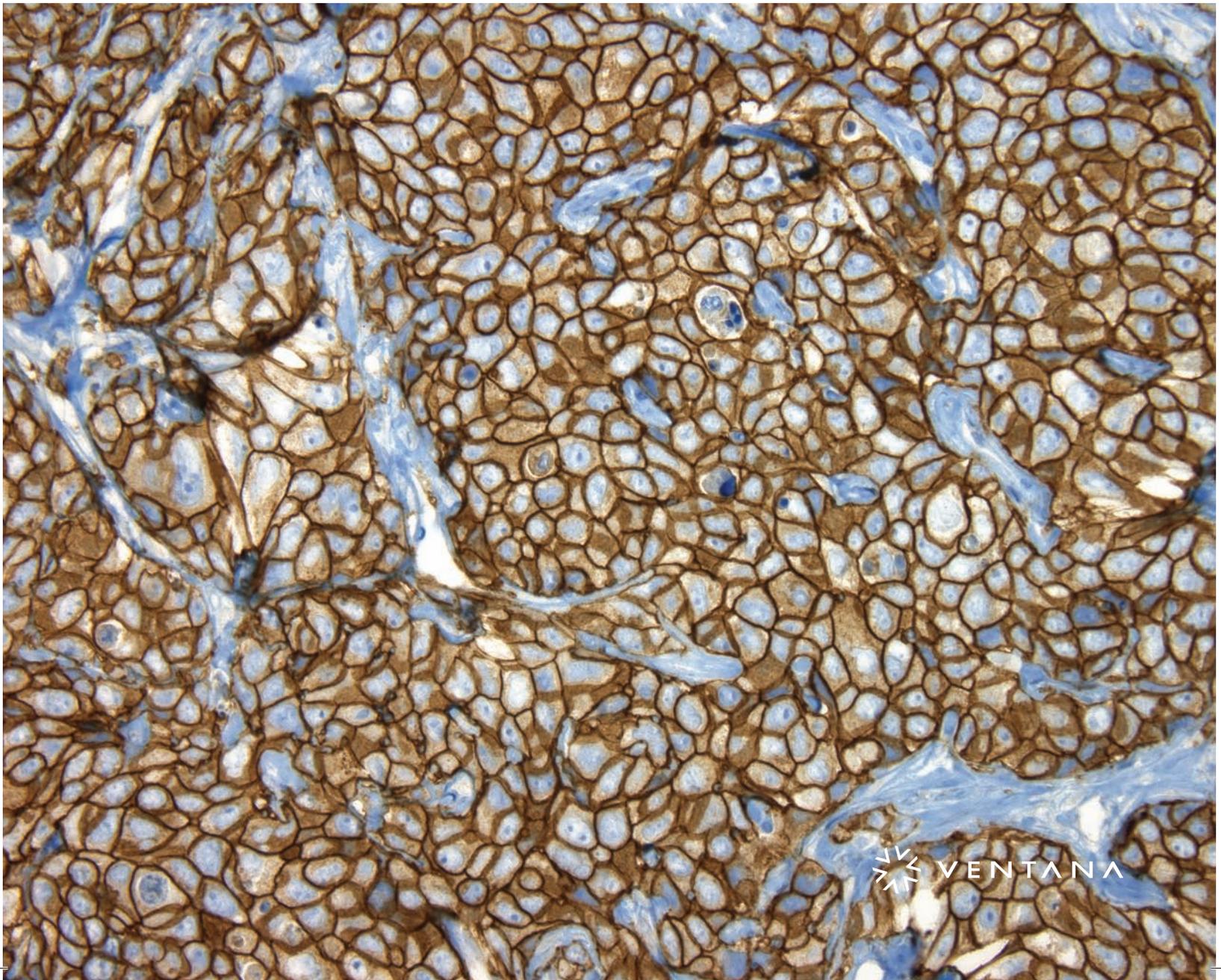


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Introduction

VENTANA anti-HER2/neu (4B5) Rabbit Monoclonal Primary Antibody [VENTANA HER2 (4b5)] is intended for the semi-quantitative detection of HER2 antigen in sections of formalin-fixed, paraffin-embedded normal and neoplastic breast and gastric tissue on a VENTANA automated slide staining device.

It is indicated as an aid in the assessment of breast and gastric cancer patients for whom Herceptin treatment is considered. The results of this test should be evaluated within the context

Purpose of interpretation guide

This guide is intended to provide pathologists with a tool to facilitate interpretation of staining patterns in breast and gastric normal and neoplastic tissue using VENTANA HER2 (4B5) in accordance with product labelling.

The photomicrographs included as part of this interpretive guide are provided to illustrate the staining patterns that may

of the patient's clinical history and other diagnostic tests evaluated by a qualified pathologist.

Any staining performed in the end user's lab should be interpreted within the context of the controls run with the clinical cases at the time of evaluation.

be present in breast and gastric carcinoma cases when stained with VENTANA HER2 (4B5). These photomicrographs are intended for new users of this test to familiarize themselves to the spectrum of staining patterns they may encounter. Included are additional cases near the clinical positive/negative cut-off and staining artefacts that may prove challenging for interpretation.

Background

VENTANA HER2 (4B5) is a rabbit monoclonal antibody (clone 4B5) directed against the internal domain of the c-erbB-2 oncoprotein (HER2). HER2 oncoprotein was cloned and characterized by Akiyama et al in 1986. It is an approximately 185 kD transmembrane glycoprotein which is structurally similar to epidermal growth factor receptor (EGFR). The protein is associated with tyrosine kinase activity similar to that of several growth factor receptors, and to that of the transforming proteins of the src family. The coding sequence is consistent with an

extracellular binding domain and an intracellular kinase domain. This suggests that HER2 may be involved in signal transduction and stimulation of mitogenic activity.¹

Clone 4B5 has been shown to react with a 185 kD protein from SK-BR-3 cell lysates via Western blotting. SK-BR-3 is a breast carcinoma cell line which has a 128-fold over expression of HER2 mRNA. The size of the band identified correlates well with that reported by Akiyama et al for HER2 protein (185 kD).¹

Clinical significance

Breast cancer is the most common carcinoma occurring in women, and the second leading cause of cancer-related death.² Early detection and appropriate treatment therapies can significantly affect overall survival.³

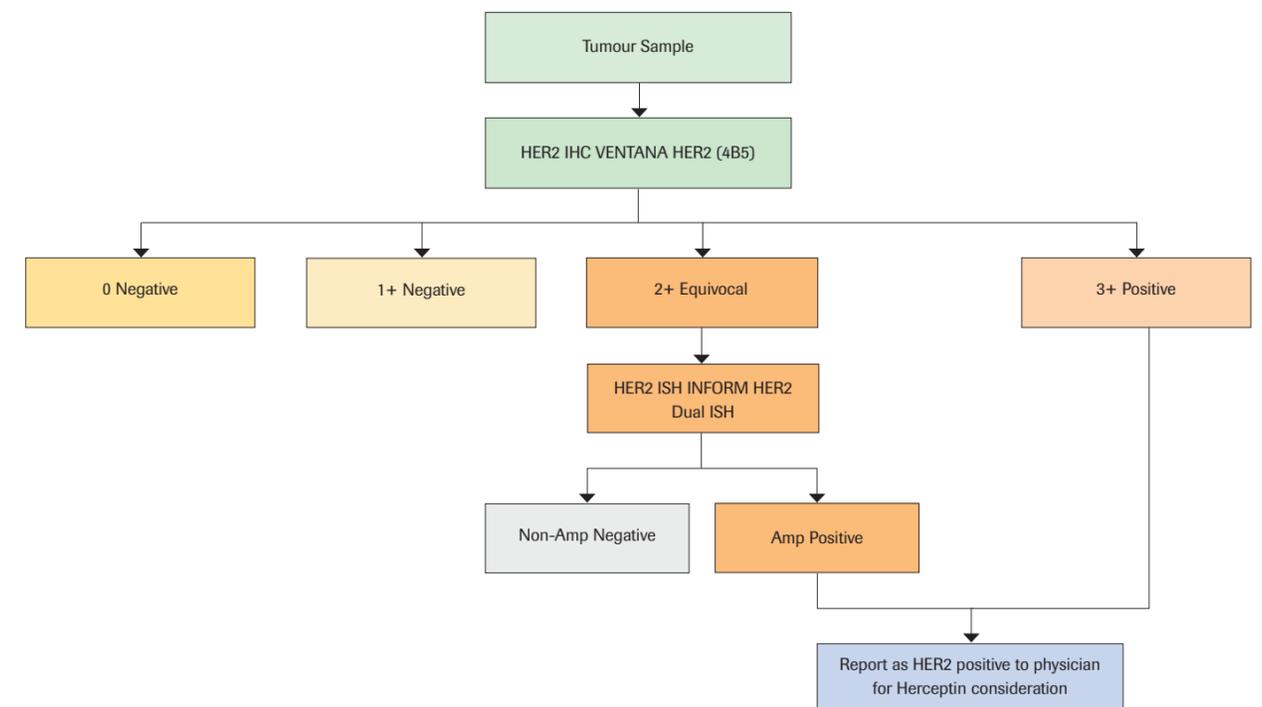
Gastric cancer is the fourth most common cancer and the second leading cause of cancer death globally. Surgery is the most common treatment for stomach cancer. However, most gastric cancer cases are, unfortunately, detected at an advanced stage and surgery is often difficult to perform. Chemotherapy is used for treating advanced gastric cancer even though the survival of cancer patients is very low.⁴

HER2 is a transmembranous protein closely related to EGFR and, like EGFR, has tyrosine kinase activity. Gene amplification and the corresponding overexpression of HER2 has been found in a variety of tumours, including breast and gastric carcinomas.^{5,6}

The therapeutic drug Herceptin has been shown to benefit some breast and gastric carcinoma patients. The drug is a humanized monoclonal antibody that binds to HER2 protein on cancer cells. Thus only patients with HER2 positive carcinomas should benefit from treatment with Herceptin. *In vitro* diagnostics for the determination of HER2 status in breast or gastric carcinomas are important to aid the clinician in determination of therapy with Herceptin.⁷

Immunohistochemistry and *in situ* hybridization are methods included in the HER2 testing algorithm (Figure 1) for determination of HER2 status as an aid in selecting patients for Herceptin therapy. VENTANA HER2 (4B5) and INFORM HER2 Dual ISH are approved products for this application in both breast and gastric cancer.

Figure 1. HER2 testing algorithm



Pre-analytical conditions affecting the performance of VENTANA HER2 (4B5)

Tissue Collection

Both surgical specimens and biopsy samples of breast and gastric tissue are acceptable for HER2 testing using VENTANA HER2 (4B5).

Fixation and Embedding

For VENTANA HER2 (4B5), the recommendation is that tissue be fixed in 10% neutral buffered formalin (NBF). The amount used is 15 to 20 times the volume of tissue. No fixative will penetrate more than 2 to 3 mm of solid tissue or 5 mm of porous tissue in a 24 hour period. A 3 mm or smaller section of tissue should be fixed no less than 4 hours and no more than 8 hours. For gastric resection specimens 18-24 hours is recommended and 6-8 hours for gastric biopsy specimens. Fixation can be performed at room temperature (15-25°C).⁸

Fixed tissues should be embedded in new paraffin.

Prolonged incubation in molten paraffin should be avoided, as high temperatures can degrade the epitope.

Properly fixed and embedded tissues expressing the antigen will remain stable for at least 2 years if stored in a cool location (15-25°C).

Sectioning

Approximately 5 µm thick sections should be cut and picked up on glass slides. The slides should be Superfrost Plus or equivalent. In-house experiments indicate that air dried cut tissue and cell line sections stored at 2-8°C are stable for at least 6 months. Each laboratory should validate the cut slide stability for their procedures and environmental storage conditions.

Addressing Variability

Variable results may occur as a result of prolonged fixation or special processes, such as decalcification of specimens containing bone.

While strict implementation of fixation condition is possible, 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 antibody binding, blocking, amplification and detection chemistries. These options enable further optimization of the assay, as needed for specific specimens.

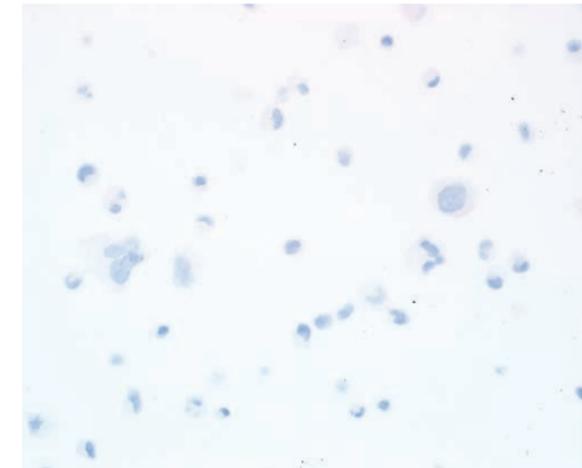
The use of pre-diluted VENTANA HER2 (4B5) and ready-to-use iVIEW or ultraVIEW DAB Detection Kits, in combination with a VENTANA automated slide stainer, reduces the possibility of human error and inherent variability resulting from individual reagent dilution, manual pipetting, and manual reagent application.

For further information and troubleshooting refer to the Package Insert, the automated slide stainer Operator's Manual or contact your local customer support representative.

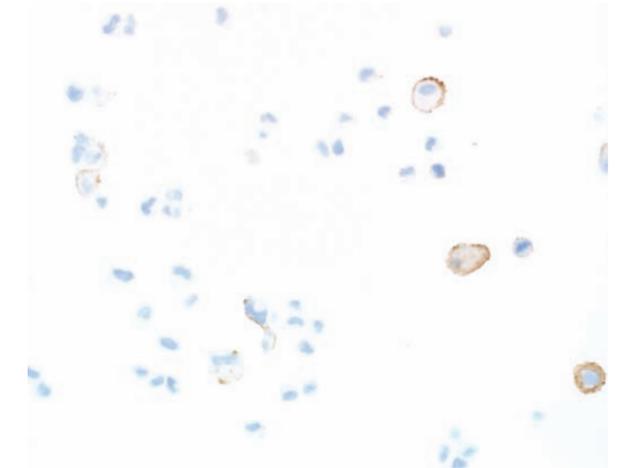
Use of cell line controls

PATHWAY HER2 4-in-1 Control Slides consist of formalin-fixed, paraffin-embedded cultured human breast cancer cell lines. They are useful for preliminary validation of the processing method used for staining slides with VENTANA HER2 (4B5) on a VENTANA BenchMark automated slide staining instrument. The clinical interpretation of any staining must be made by a qualified pathologist.

Copy numbers for each control are an average of 3 lots of PATHWAY HER2 4-in-1 Control Slides.



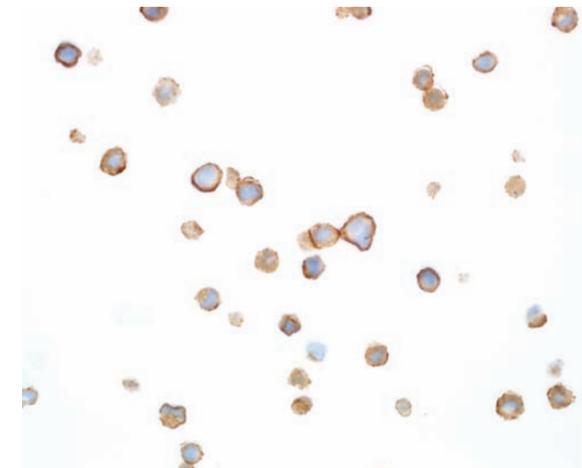
(40X)
The Level 0 Cell Line Control (MDA-MB-231) is scored negative when processed appropriately.



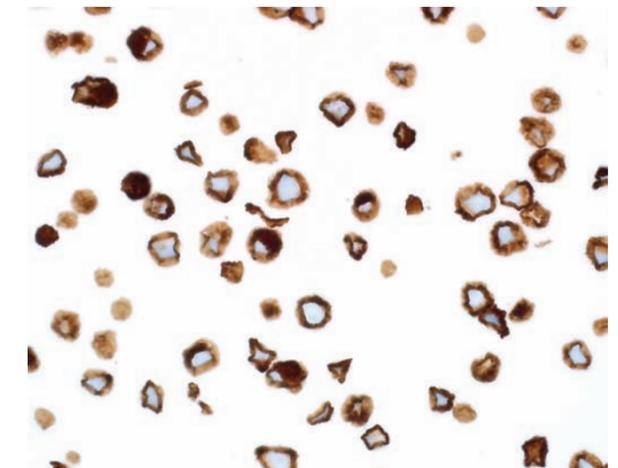
(40X)
The Level 1+ Cell Line Control (T-47D) stains at an intensity level of 1+.

Note: because HER2 antigen is not uniformly coating the surface of these cells, not all cross sections will stain. When first evaluating this cell line, scan the entire cell field. It may also be necessary to examine it at higher magnification (40X obj) to pick up the 1+ staining in the scattered cells. When processed appropriately, >10% of the cells will stain with 1+ intensity.

If the Level 1+ control does not stain appropriately, the staining run should be repeated.



(40X)
The Level 2+ Control Cell Line (MDA-MB-453) stains at an intensity level of 2+ with complete "ring" pattern in >10% of the cells. In contrast to 3+ cases, the staining scored as 2+ has a crisper and more clearly delineated ring, while cases scored as 3+ exhibit a very thick outline (compare to Level 3+ Control Cell Line).



(40X)
The Level 3+ Cell Line Control (BT-474) is a high expression cell line that stains at an intensity level of 3+ in approximately 100% of the cells.

Interpretation of staining results in breast cancer

Breast carcinomas that are considered positive for HER2 protein overexpression must meet a threshold criteria for the intensity and pattern of membrane staining (3+ on a scale of 0 to 3+), and for the percent positive tumour cells (greater than 10%).

Staining must localize to the cell membrane and be circumferential (complete). Staining of the cytoplasm may be present, but this staining is not included in the determination of positivity (Table 1).

Table 1. Criteria for intensity and pattern of cell membrane staining in breast carcinoma

Staining Pattern	Score (Report to requesting physician)	HER2 Staining Assessment
No membrane staining is observed	0	Negative
Faint, partial staining of the membrane in any proportion of the cancer cells	1+	Negative
Weak to moderate complete staining of the membrane, greater than 10% of cancer cells	2+	Equivocal*
Strong, complete staining of the membrane greater than 10% of cancer cells	3+	Positive

*Recommend reflex to ISH

Identification of appropriate staining pattern

Membranous Staining

The HER2 protein is expressed in the cell membrane of both normal and neoplastic human breast tissues. The HER2 protein is expressed at a level detectable by immunohistochemistry in 15 to 30 percent of invasive ductal cancers.⁹

Using frozen tissue sections Press et al. reported weak staining of normal epithelial cells in the gastro-intestinal, respiratory, reproductive, and urinary tract as well as in the skin, breast, and placenta.⁹ The levels of HER2 protein expression in normal tissues were similar to those observed in non-amplified, non-overexpressing breast cancers. The weak membrane staining observed in frozen tissue sections of normal tissue was frequently absent in the corresponding formalin-fixed

paraffin section. Intense staining of the cell membrane was found only in the tumour cells of invasive breast carcinoma.

If intense staining of normal epithelium is observed the slide should be considered inappropriate for interpretation and further optimization and re-staining is required.

Cytoplasmic Staining

Cytoplasmic staining in the absence of membrane staining was not observed by Press et al. Taylor et al. reported that cytoplasmic staining for HER2 protein is not associated with the presence of detectable HER2 mRNA in breast cancer.¹⁰ Cytoplasmic only staining in breast cancer is not known to be clinically relevant.

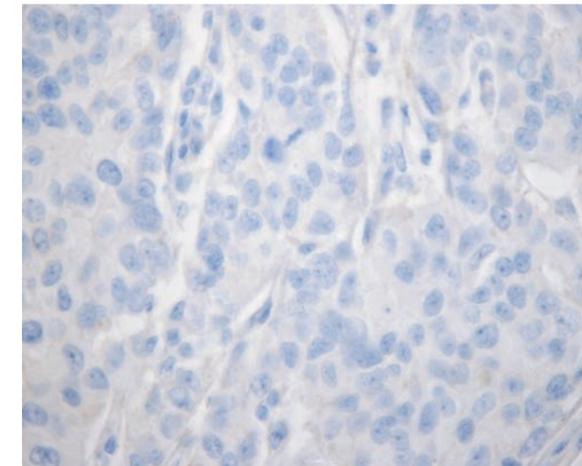
Evaluating pattern and intensity of staining

The breast tissue section should be examined for pattern and intensity of staining including determination of completeness of the cell membrane stain. Staining that completely encircles the cell membrane should be scored as "2+" or "3+."

Partial, incomplete staining of the membrane should be scored

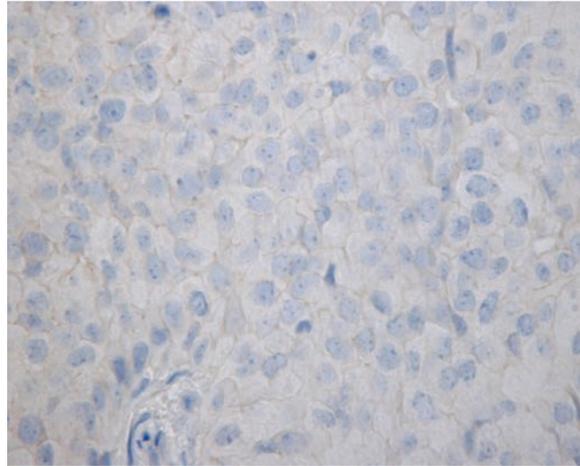
as "1+." It may be necessary to examine some cases at 40X or higher magnification to discriminate between 0 or 1+ and 1+ or 2+. Cytoplasmic and/or nuclear staining should not be factored into determination of positivity.

Photoset A: Negative Cases, Score 0

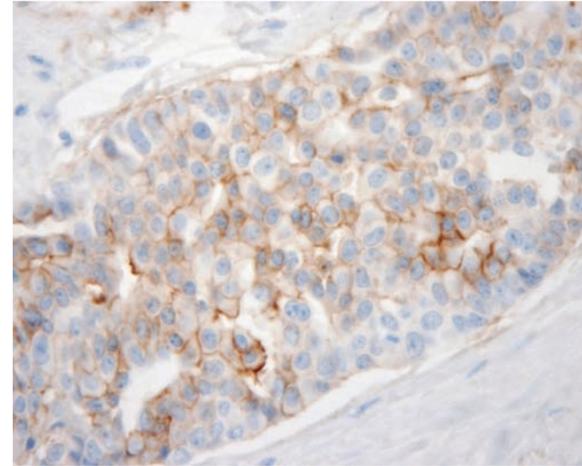


Case 1 40X magnification
Invasive breast carcinoma, Score 0

Photoset B: Negative Cases, Score 1+

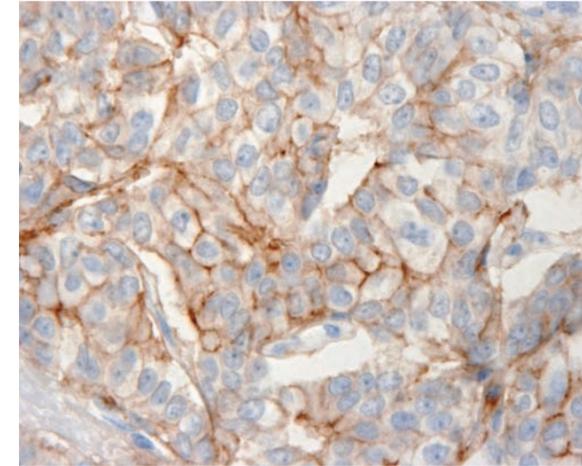


Case 2 40X magnification
Invasive breast carcinoma, Score 1+

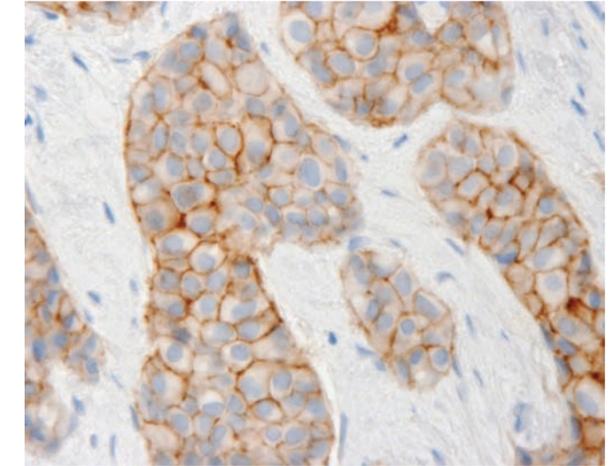


Case 3 40X magnification
Invasive breast carcinoma, Score 1+

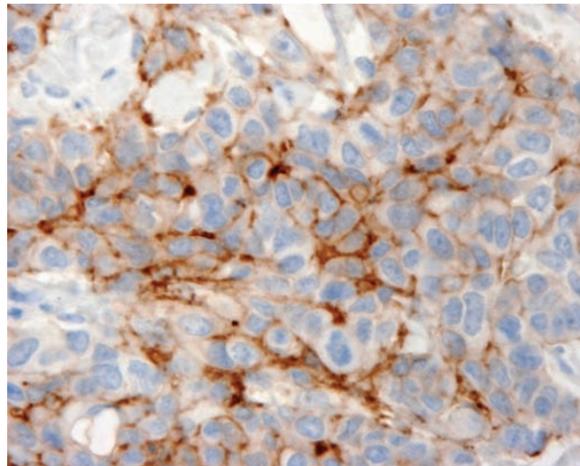
Photoset C: Equivocal Cases, Score 2+



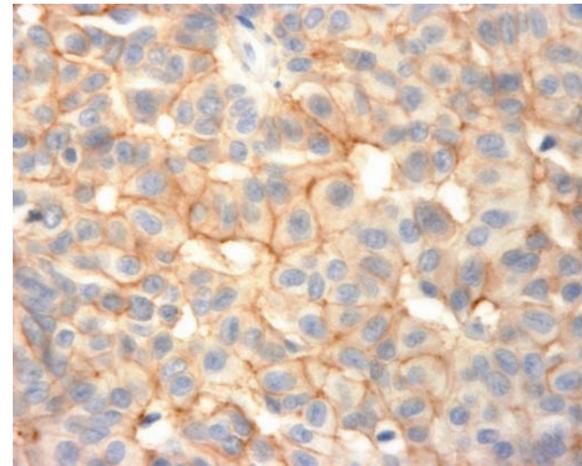
Case 6 40X magnification
Invasive breast carcinoma, Score 2+



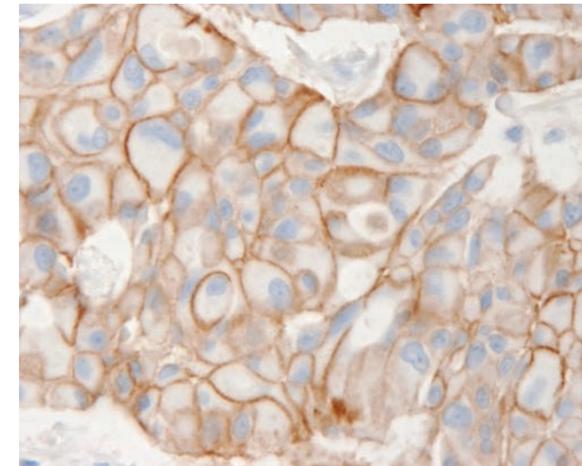
Case 7 40X magnification
Invasive breast carcinoma, Score 2+



Case 4 40X magnification
Invasive breast carcinoma, Score 1+



Case 5 40X magnification
Invasive breast carcinoma, Score 1+



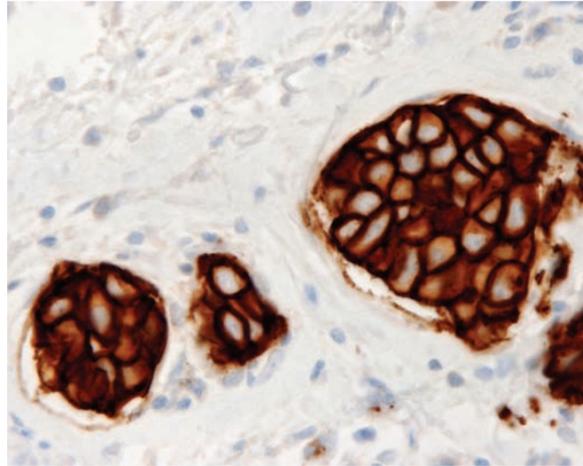
Case 8 40X magnification
Invasive breast carcinoma, Score 2+

These cases illustrate a cell membrane staining pattern that is scored as 1+. Cell membrane staining is partial rather than a full "ring" pattern. The intensity of the membrane staining varies, but the pattern is predominantly incomplete ring staining.

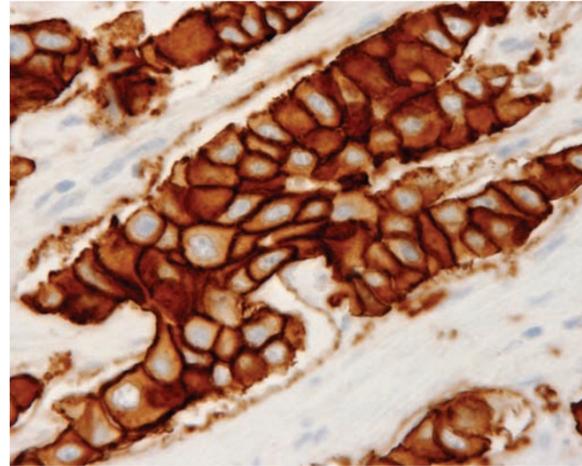
Diffuse cytoplasmic staining can also be present. These cases would be considered negative. Scattered cells with the full ring (2+) pattern can be present.

These three cases illustrate staining that is scored as 2+. Cell membrane staining demonstrates the complete "ring" pattern in and with the exception of Case 6, in the majority of cells. In contrast to 3+ cases, the staining scored as 2+ has a crisper and more clearly delineated ring, while cases scored as 3+ exhibit a very thick, almost folded outline (compare to Photoset D). Weak, diffuse cytoplasmic staining can also be present in contrast to 3+ cases where cytoplasmic staining is often intense. Cases scored as 2+ often display staining heterogeneity with focal areas of 1+ and/or 3+ staining intermixed with the 2+ cancer cells.

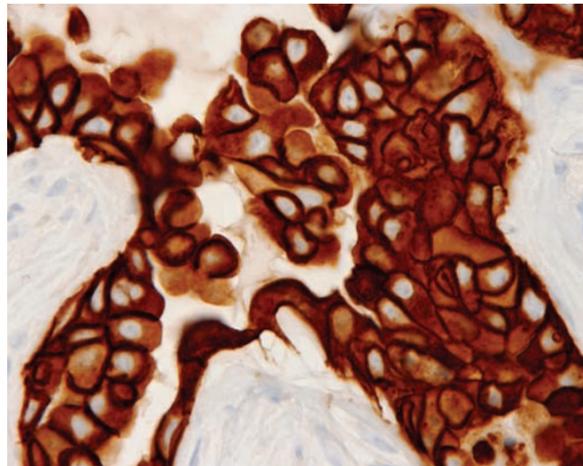
Photoset D: Positive Cases, Score 3+



Case 9 40X magnification
Invasive breast carcinoma, Score 3+



Case 10 40X magnification
Invasive breast carcinoma, Score 3+



Case 11 40X magnification
Invasive breast carcinoma, Score 3+

These cases illustrate cell membrane staining that is scored as 3+. Cell membrane staining is very intense, thickly outlined, and demonstrates the complete "ring" pattern in the majority of the cells. Strong, diffuse cytoplasmic staining is often also present. Cases scored as 3+ often have approximately 100% of cells with intense positive membrane staining.

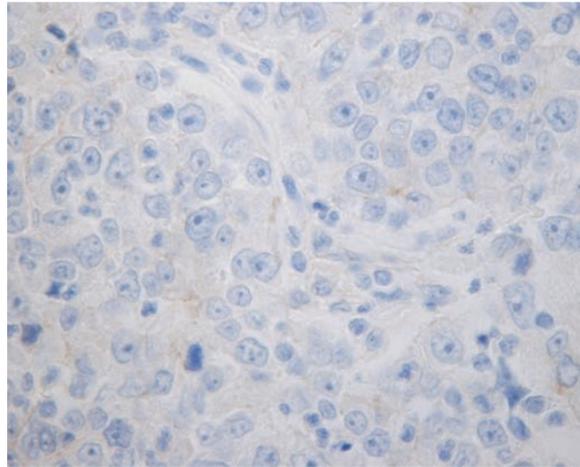
Interpreting borderline cases

The most difficult area of interpretation is cases that fall on the borderline between an intensity level of "1+" and "2+", or where there is a mixture of different expression levels. Here are some tips for handling these cases:

1. Evaluate the borderline case within the context of unambiguous "1+" and "2+" cases to regain perspective. These can be the photomicrographs contained within this Interpretation Guide or control tissue.
2. Remember that pattern plays a primary role in the score. Only complete membrane "rings" are scored as 2+.
3. Scan complete tissue section to ensure scoring in well-preserved and well stained areas only.
4. Consider repeating the staining on another section or repeat staining on sections from a different block if none of the above suggestions resolve the diagnosis. Lewis et al. reported that 2+ cases often show significant intratumoural heterogeneity and that staining of additional sections can yield results that provide improved correlation with HER2 gene status.
5. If a result remains in question consider alternative testing methods such as *in situ* hybridization.

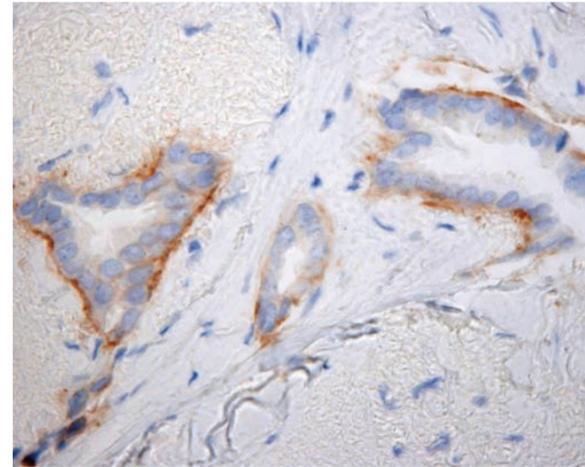
Photoset E: Borderline Patterns

Photoset E provides examples that are representative potential borderline (1+ versus 2+) cases, with an emphasis on examples close to the 10% cut-off for positivity.



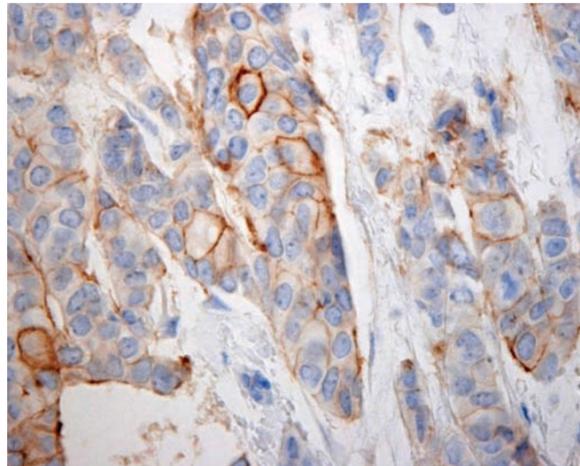
Case 12 40X magnification
Invasive breast carcinoma, Score 0

This case is completely negative and provided for comparative purposes.



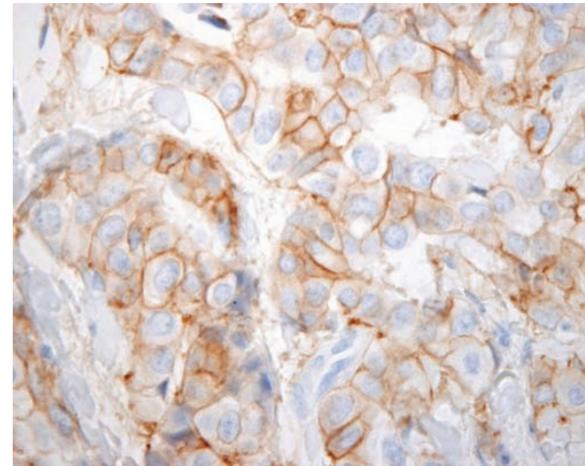
Case 13 40X magnification
Invasive breast carcinoma, Score 0

This field demonstrates cell membrane staining in a tubular carcinoma but only along the basal cell surface. Partial staining of lateral or apical surfaces is not present.



Case 14 40X magnification
Invasive breast carcinoma, Score 1+

Membrane staining: this case demonstrates 2+ cell membrane and partial membrane staining in of cancer cells. When close to the cut-off point, it may be necessary to perform actual cell counts. Three representative fields at 40X or greater magnification should be chosen, and 100 tumour cells per field counted.



Case 15 40X magnification
Invasive breast carcinoma, Score 2+

This field demonstrates 2+ cell membrane staining (circumferential, thin ring) in greater than 10% of tumour cells and the staining pattern is heterogeneous with intermixed 1+ and 3+ cells.

Interpretation of staining results in gastric cancer

Gastric carcinomas that are considered positive for HER2 protein overexpression must meet a threshold criteria for the intensity and pattern of membrane staining (3+ on a scale of 0 to 3+), and for the percent positive tumour cells. Staining must localize to the cell membrane but need not be completely circumferential, as basolateral staining is regularly observed and should be

considered for scoring. Staining of the cytoplasm and/or the nucleus may be present, but this staining is not included in the determination of positivity (Figure 2). In gastric carcinoma the percentage of positive tumour cells depends upon whether the sample is a biopsy specimen (≥5 cohesive cells) or resection specimen (≥10%) (Table 2).¹¹

Table 2. Criteria for intensity and pattern of cell membrane staining in gastric carcinoma

Staining pattern - resection specimen	Staining pattern - biopsy specimen	Score (report to requesting physician)	HER2 staining assessment
No reactivity or membranous reactivity in <10% of tumour cells	No reactivity or membranous reactivity in any tumour cell	0	Negative
Faint / barely perceptible membranous reactivity in ≥10% of tumour cells; cells are reactive only in part of their membrane	Tumour cell cluster* with a faint / barely perceptible membranous reactivity irrespective of percentage of tumour cells stained	1+	Negative
Weak to moderate complete, basolateral or lateral membranous reactivity in ≥10% of tumour cells	Tumour cell cluster with a weak to moderate complete, basolateral or lateral membranous reactivity irrespective of percentage of tumour cells stained	2+	Equivocal**
Strong complete, basolateral or lateral membranous reactivity in ≥10% of tumour cells	Tumour cell cluster with a strong, complete basolateral or lateral membranous reactivity irrespective of percentage of tumour cells stained	3+	Positive

*≥5 cohesive cells

** Recommend reflex to ISH

Identification of appropriate staining pattern

Membranous Staining

The HER2 protein is expressed in the cell membrane of both normal and neoplastic human tissues. The HER2 protein is expressed at a level detectable by immunohistochemistry in up to 30% of intestinal type, 15% mixed type and 5% diffuse type gastric cancers.¹¹

In establishing the scoring guidelines for HER2 immunohistochemistry in gastric cancer Hoffmann et al. note that while strong membranous staining is evidence of HER2 protein overexpression in neoplastic cells it need not be completely circumferential.¹¹

Cytoplasmic And Nuclear Staining

Rüschhoff et al. reported diffuse cytoplasmic staining with or without nuclear staining in gastric cancer. Only membranous staining should be used in determination of HER2 protein expression in gastric cancer.¹²

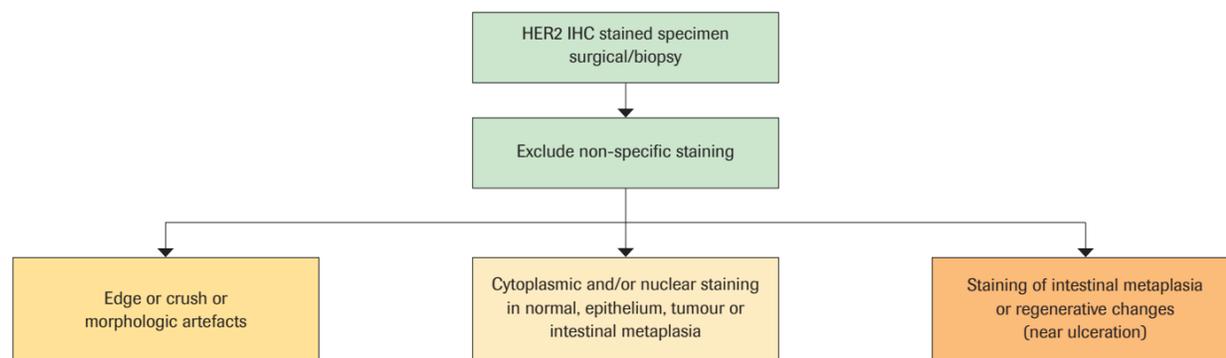
Immunohistochemical staining with the anti HER2/neu clone 4B5 can produce cytoplasmic and nuclear staining of normal gastric mucosa and more infrequently of neoplastic cells in gastric carcinoma and gastric/oesophageal carcinoma. The nature of this cytoplasmic and nuclear staining is currently unknown. This staining pattern should not be confused with the discrete membranous staining that is indicative of HER2 positivity in neoplastic cells.

Evaluating pattern and intensity of staining

Gastric carcinomas that are considered positive for HER2 protein overexpression must meet a threshold criteria for the intensity and pattern of membrane staining (3+ on a scale of 0 to 3+), and for the percent positive tumour cells (greater than 10% in surgical specimens or at least 5 cohesive cells in biopsy specimens).

Staining must localize to the cell membrane and can be circumferential (complete) or basolateral or lateral (incomplete). Staining of the cytoplasm and nucleus may be present, but this staining is not included in the determination of positivity (Figure 2).

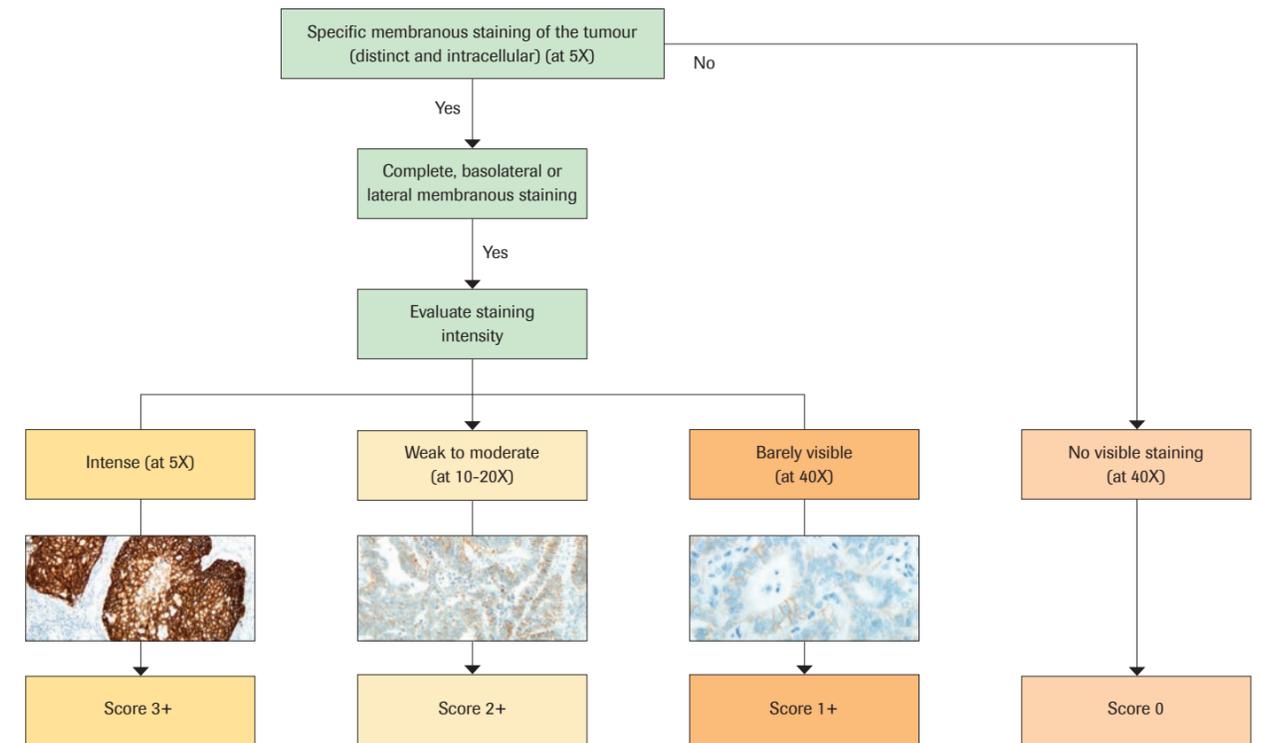
Figure 2. HER2 IHC sample exclusion criteria for gastric and oesophageal junction samples



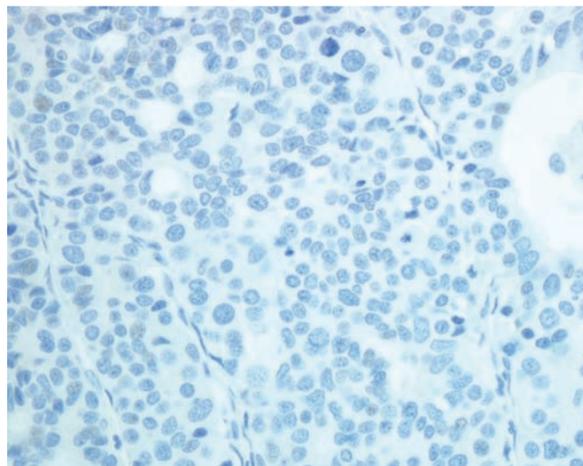
Staining intensity can be assessed at different magnifications to guide the classification. The strong HER2 positive, 3+, staining can easily be observed at 5X, while membranous staining

confirmed at 10X or 20X points to HER2 2+. 40X points to HER2 0 or 1+ score (Figure 3).

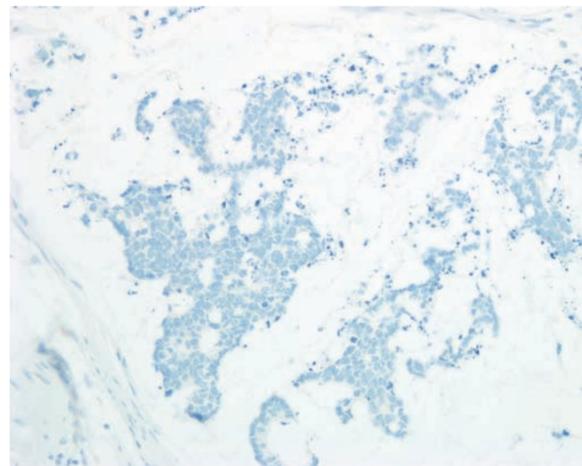
Figure 3. HER2 IHC classification in gastric and oesophageal junction samples



Photoset F: Negative Cases, Score 0

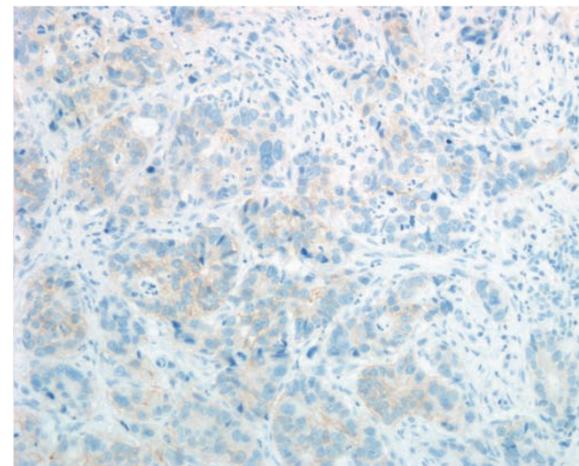


Case 16 40X magnification
Invasive gastric adenocarcinoma, score 0

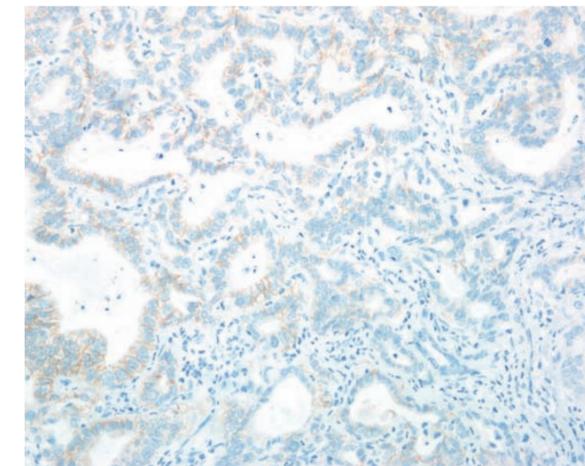


Case 17 20X magnification
Invasive gastric adenocarcinoma, score 0

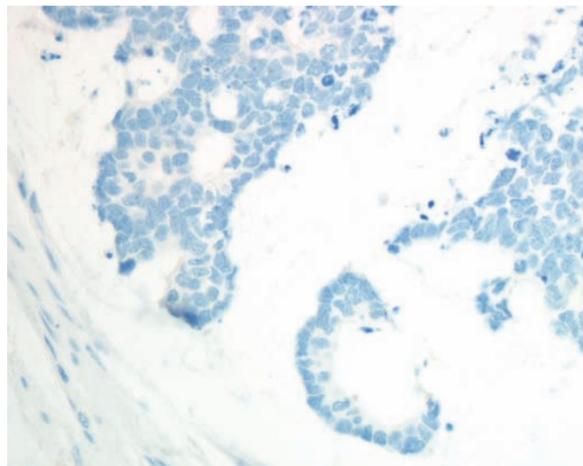
G: Negative Cases, Score 1+



Case 19 20X magnification
Invasive gastric adenocarcinoma, score 1+

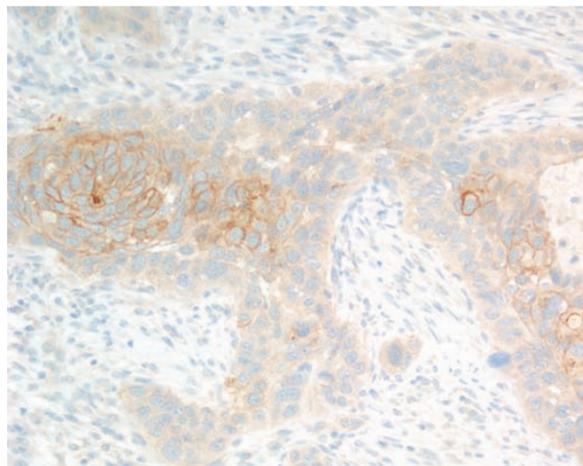


Case 20 20X magnification
Invasive gastric adenocarcinoma, score 1+

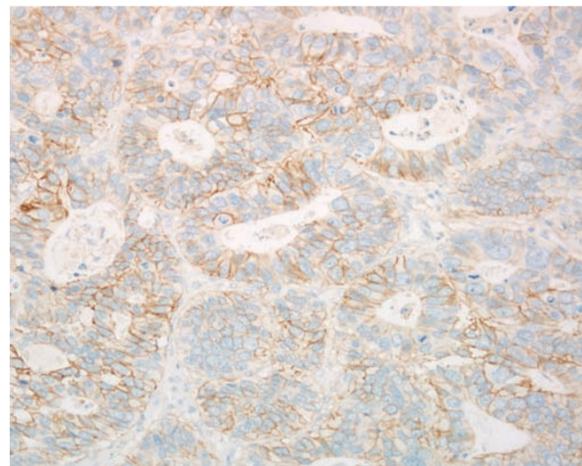


Case 18 40X magnification
Invasive gastric adenocarcinoma, score 0

Photoset H: Equivocal Cases, Score 2+

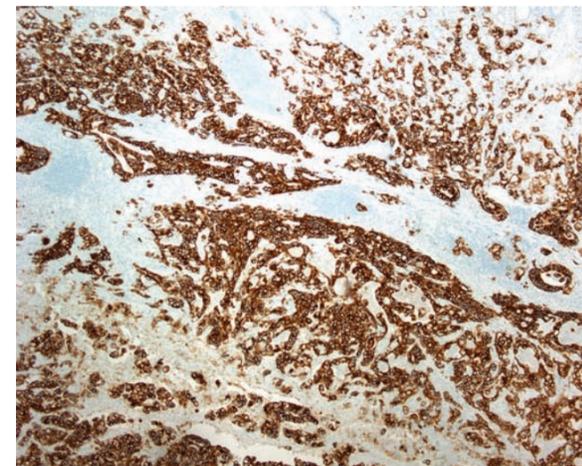


Case 21 20X magnification
Invasive gastric adenocarcinoma, score 2+

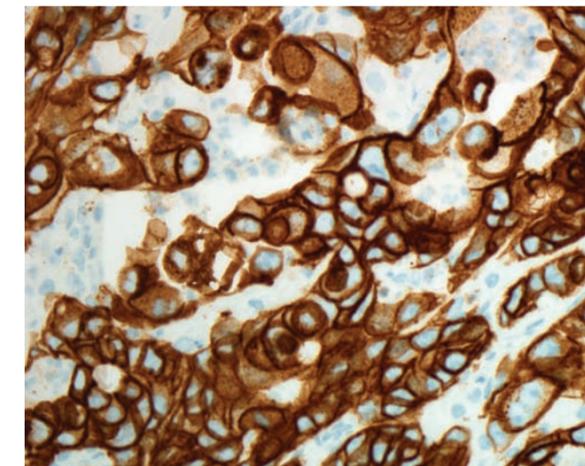


Case 22 20X magnification
Invasive gastric adenocarcinoma, score 2+

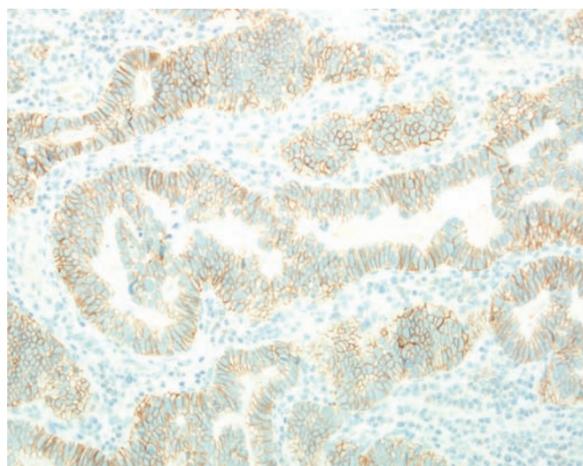
Photoset I: Positive Cases, Score 3+



Case 24 5X magnification
Invasive gastric adenocarcinoma, score 3+



Case 25 40X magnification
Invasive gastric adenocarcinoma, score 3+, with cytoplasmic staining



Case 23 20X magnification
Invasive gastric adenocarcinoma, score 2+

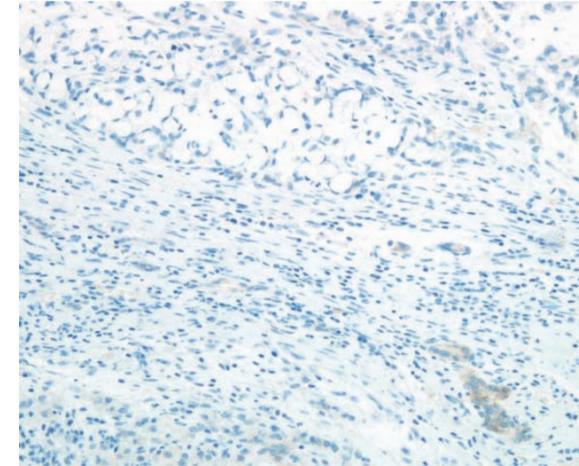
Interpreting borderline cases

The most difficult area of interpretation is cases that fall on the borderline between an intensity level of "1+" and "2+", or where there is a mixture of different expression levels. Here are some tips for handling these cases:

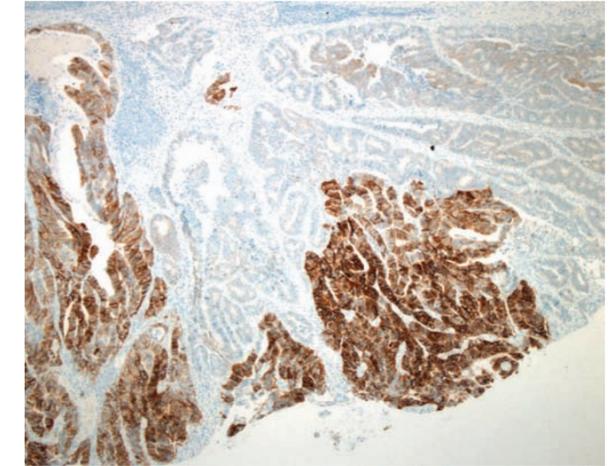
1. Evaluate the borderline case within the context of unambiguous "1+" and "2+" cases to regain perspective. These can be the photomicrographs contained within this Interpretation Guide or control tissue.
2. Remember that pattern plays a primary role in the score. Both complete and incomplete membranous staining can be considered in gastric cancer. Refer to "Determining HER2 staining intensity in gastric and oesophageal junction samples", Figure 3 & 4 as guidance.

3. Scan complete tissue section to ensure scoring in well-preserved and well stained areas only.
4. Consider repeating the staining on another section or repeat staining on sections from a different block if none of the above suggestions resolve the diagnosis.
5. If a result remains in question consider alternative testing methods such as *in situ* hybridization.

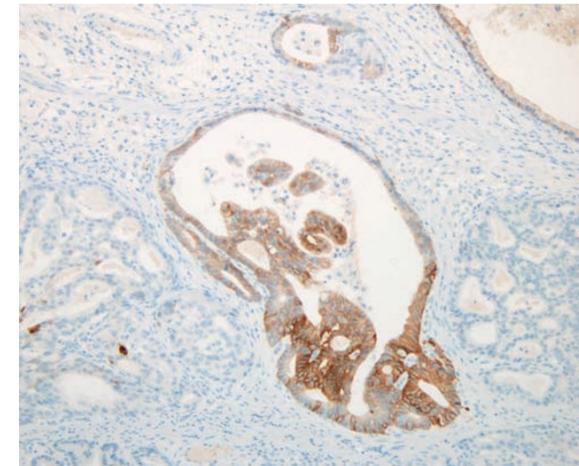
Photoset J: Borderline Patterns



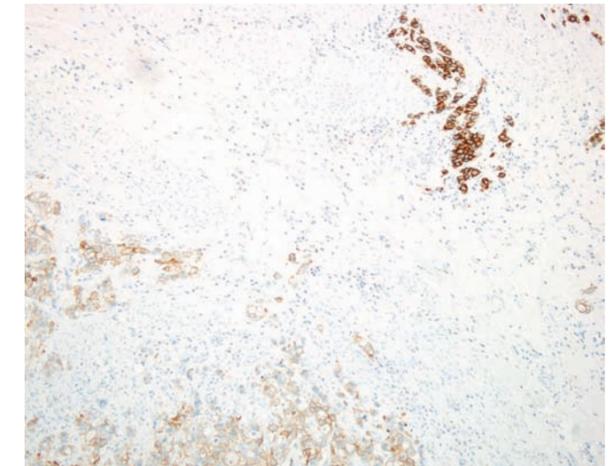
Case 26 20X magnification
Invasive gastric adenocarcinoma, score 0/1+ heterogeneous for staining intensity and morphology. Top left is an example of signet ring morphology with lower right an example of more glandular morphology.



Case 27 5X magnification
Invasive gastric adenocarcinoma, with heterogeneity

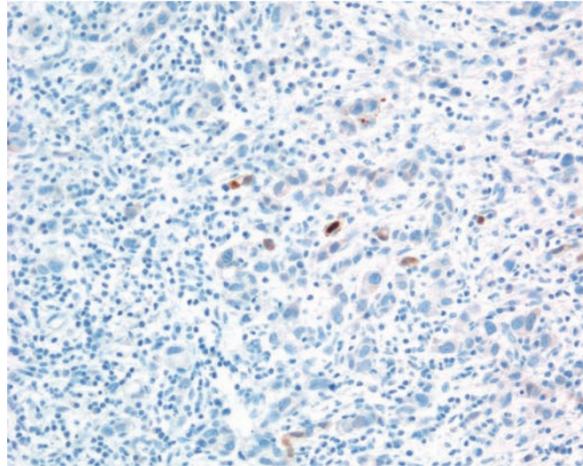


Case 28 10X magnification
Invasive gastric adenocarcinoma, with heterogeneity

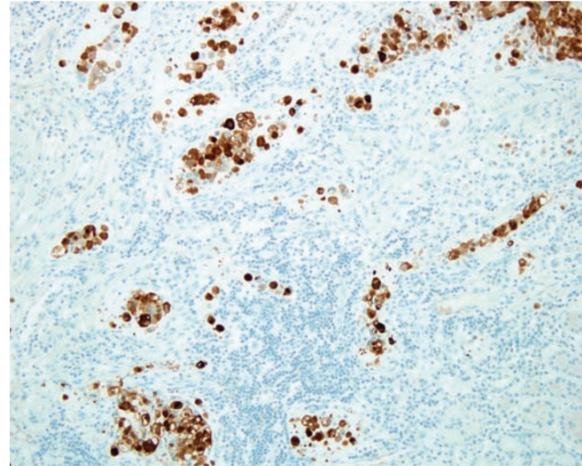


Case 29 10X magnification
Invasive gastric adenocarcinoma, with heterogeneity

Photoset J: Borderline Patterns (continued)

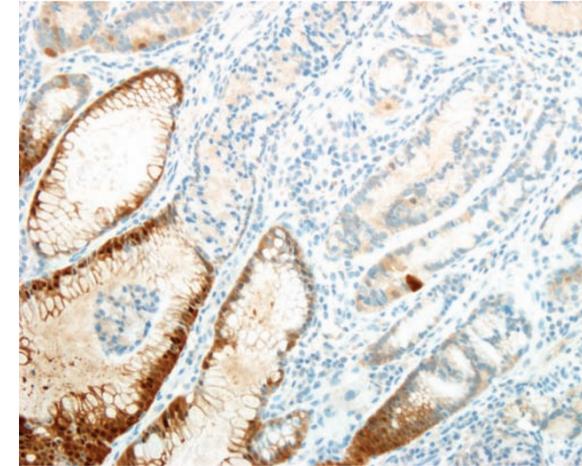


Case 30 20X magnification
Invasive gastric adenocarcinoma, with nuclear staining, score 0

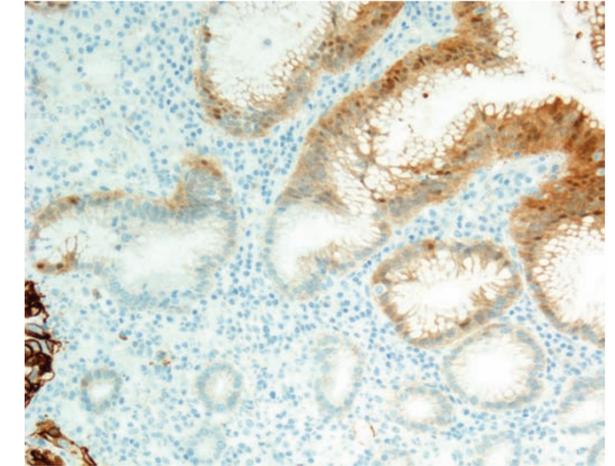


Case 31 10X magnification
Invasive gastric adenocarcinoma, with nuclear and cytoplasmic staining, score 2+

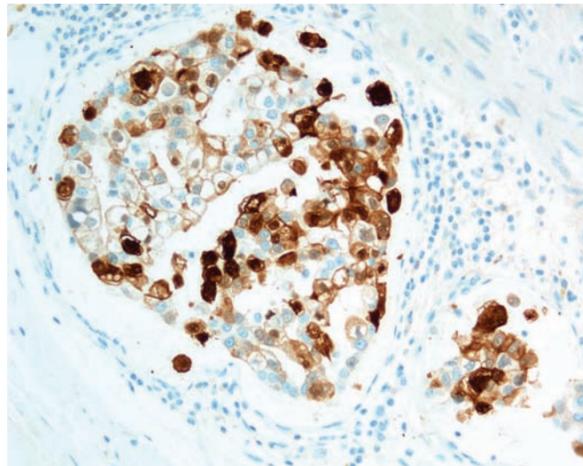
Photoset J: Borderline Patterns (continued)



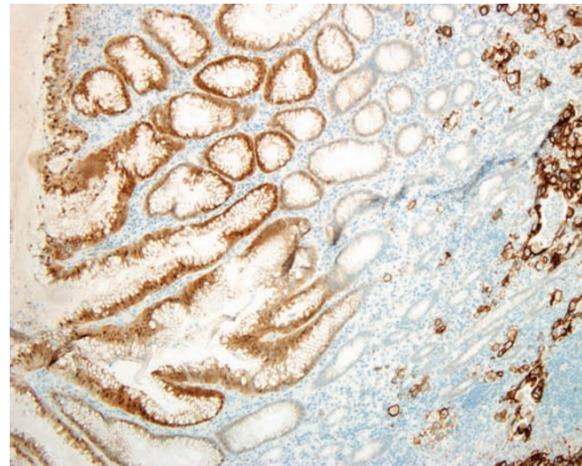
Case 34 20X magnification
Cytoplasmic and nuclear staining artefact in normal mucosa



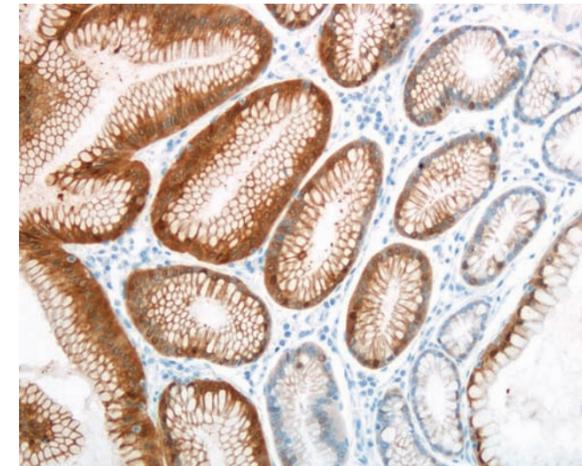
Case 35 20X magnification
Cytoplasmic and nuclear staining artefact in normal mucosa



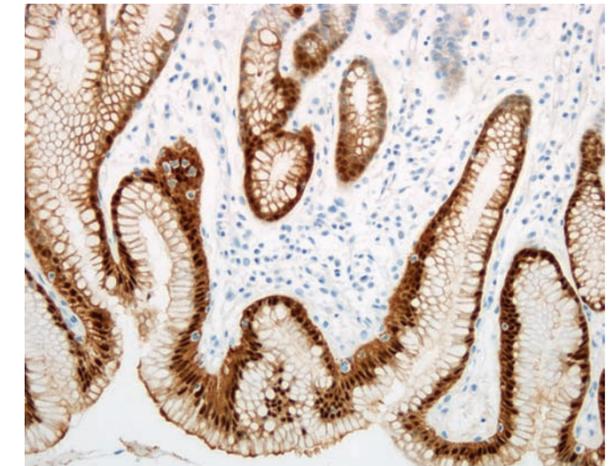
Case 32 20X magnification
Invasive gastric adenocarcinoma, with nuclear and cytoplasmic staining, score 2+



Case 33 10X magnification
Invasive gastric adenocarcinoma, with adjacent normal mucosa having cytoplasmic and nuclear staining, score 3+



Case 36 20X magnification
Cytoplasmic and nuclear staining artefact in normal mucosa



Case 37 20X magnification
Cytoplasmic and nuclear staining artefact in normal mucosa

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