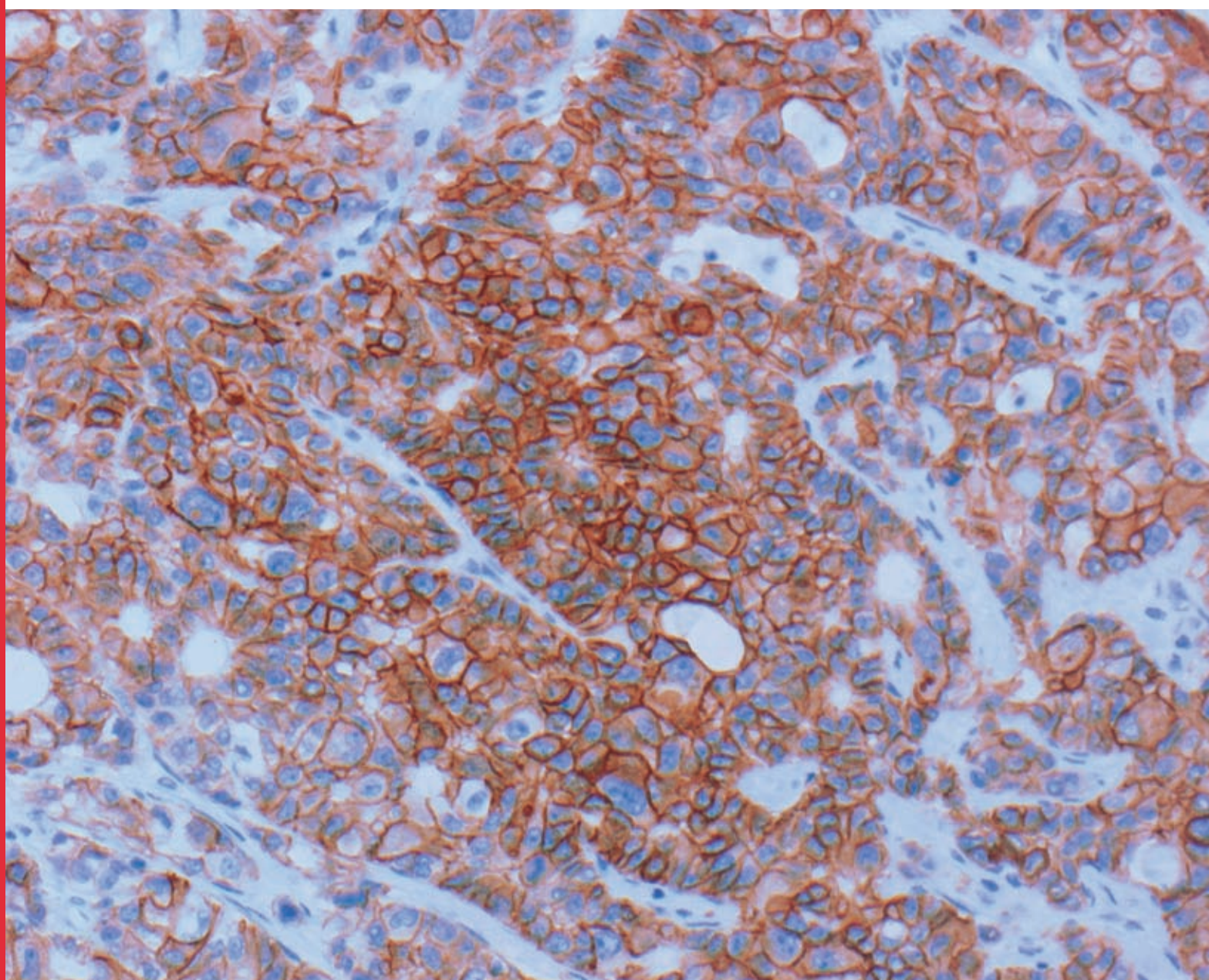
PATHOLOGY

HercepTest™ | Interpretation Manual - Gastric Cancer



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Introduction

HercepTest™ Interpretation Manual

HercepTest™ is a semi-quantitative immunohistochemical assay for determination of HER2 protein (c-erbB-2 oncoprotein) overexpression in breast cancer tissues routinely processed for histological evaluation and in formalin-fixed, paraffin-embedded cancer tissue from patients with adenocarcinoma of the stomach, including gastroesophageal junction.

HercepTest™ is an aid in the assessment of patients for whom treatment with humanized monoclonal antibody to HER2 protein, Herceptin™ (trastuzumab), is being considered. Decision regarding Herceptin™ treatment should be made within the context of the patient's clinical history.

This manual is about interpretation of human FFPE stomach and gastroesophageal tissue specimens stained with HercepTest™.

HercepTest™ Interpretation Guidelines

This HercepTest™ Interpretation Manual - Gastric is provided as a tool to help guide pathologists and laboratorians to achieve correct and reproducible results. The goal of this manual is to familiarize you with the requirements for scoring stomach, including gastroesophageal junction adenocarcinomas stained with HercepTest™. Adenocarcinoma of the stomach including gastroesophageal junction is also referred to as gastric cancer in this document. Example cases of various HER2 scores are provided for reference. The HercepTest™ package insert guidelines will be reviewed and technical tips for ensuring high-quality staining in your laboratory will be given. Reviewing this HercepTest™ Interpretation Manual-Gastric will provide a solid foundation for evaluating slides stained with HercepTest™. Stomach or gastroesophageal junction adenocarcinomas tested for HER2 protein expression are given a score from 0 to 3+. In this manual, we will also focus on the samples that are more difficult to interpret.

HER2 FISH pharmDx™

Despite the high quality of HercepTest™, clinical response of equivocal for gastric specimens has remained an area of uncertainty within HER2 assessment. *HER2* FISH pharmDx™ complements HercepTest™ by quantitatively determining *HER2* gene amplification and clarifying equivocal cases. HercepTest™ and *HER2* FISH pharmDx™ Kit enhance patient care by aiding in proper determination of the appropriate course of treatment.

Photomicrographs

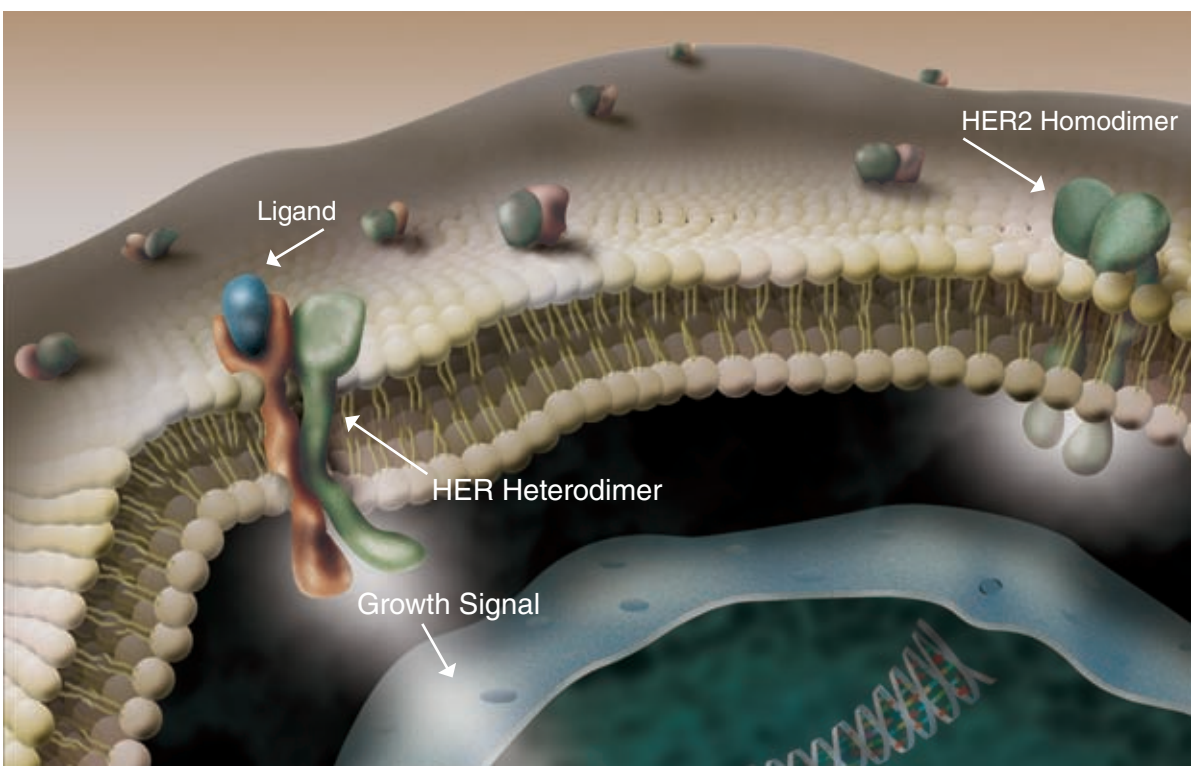
The included photomicrographs are gastric cancer unless otherwise noted.

HER2 Protein and HER Family

HER2 Overview

The gene encoding HER2 is located on chromosome 17 and is a member of the EGF/erbB growth factor receptor family, which also includes epidermal growth factor receptor (EGFR, or HER1), HER3/erbB3 and HER4/erbB4. All of these genes encode transmembrane growth factor receptors, which are tyrosine kinase type 1 receptors with growth stimulating potential. Activation of HER family members occurs when the ligand and a dimer of the same monomer or other member of the HER family are bound together, as shown in the below representation. Once activation has occurred, tyrosine autophoshorylation of cytoplasmic signal proteins transmit signals to the nucleus, thus regulating aspects of cell growth, division, differentiation and migration.

Overexpression of HER2 receptors results in receptors transmitting excessive signals for cell proliferation to the nucleus. This may lead to more aggressive growth of the transformed cell. Data supports the hypothesis that the HER2-transfected cells directly contribute to the pathogenesis and clinical aggressiveness of tumors that overexpress HER2. This overexpression is associated with poor prognosis, including reduced relapse-free and overall survival.



Representation of HER Family

HER2 Testing Algorithm

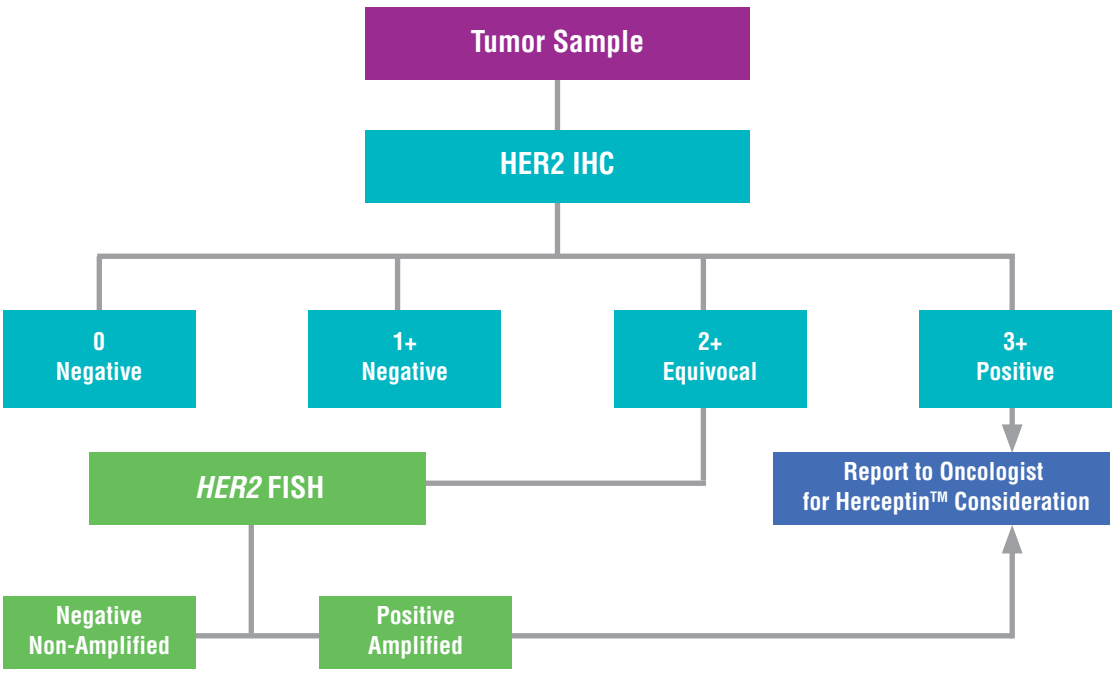
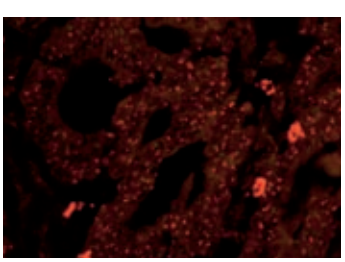


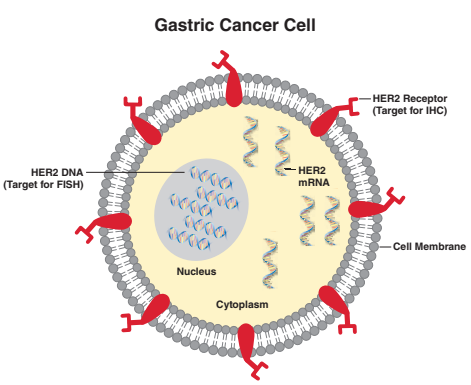
Figure 1
HER2 testing algorithm for gastric cancer. The suggested algorithm specifies that all cases are diagnosed using IHC as the primary method. Those cases that are scored as equivocal 2+ are then subsequently tested using the FISH technique to ensure that appropriate assessment of patients for whom trastuzumab treatment is being considered, is adhered to.
Laboratories performing HER2 testing should meet quality assurance standards.

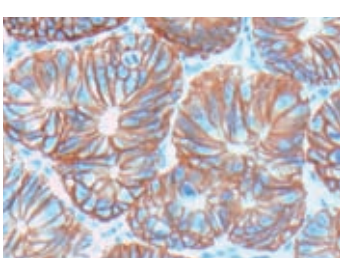
HER2 Testing IHC and FISH

Immunohistochemistry (IHC) measures the level of HER2 receptor overexpression, while fluorescence in situ hybridization (FISH) quantifies the level of *HER2* gene amplification. Together they are the most commonly used methods of determining HER2 status in routine diagnostic settings.



Amplified Result, Score > 2
Gastric cancer specimen stained with *HER2* FISH pharmDx™ Kit.





Positive Result, Score 3+
Gastric cancer specimen stained with HercepTest™.

HER2 gene amplification is the underlying biological change that results in HER2 overexpression.

Figure 2
IHC and FISH targets for HER2 testing

OVERVIEW

The HercepTest™ Kit

The HercepTest™ assay is a semi-quantitative immunohistochemical kit system for determination of HER2 protein overexpression in breast cancer tissues routinely processed for histological evaluation and in formalin-fixed, paraffin-embedded cancer tissue from patients with adenocarcinoma of the stomach, including gastroesophageal junction.

Following incubation with the primary antibody to human HER2 protein, this kit employs a ready-to-use Visualization Reagent based on dextran technology. This reagent consists of both secondary goat anti-rabbit molecules and horseradish peroxidase molecules linked to a common dextran polymer backbone, thus eliminating the need for sequential application of link antibody and peroxidase conjugate. The enzymatic conversion of the subsequently added chromogen results in formation of a visible reaction product at the antigen site. The specimen may then be counterstained and coverslipped. Control cell line slides are provided.

Three HercepTest™ kit configurations are available:

K5204	HercepTest™ for Manual use	35 Tests
K5207	HercepTest™ for the Dako Autostainer	50 Tests
SK001	HercepTest™ for Automated Link Platforms	50 Tests

HercepTest™ is a complete kit and includes:

- Peroxidase-Blocking Reagent
- Rabbit Anti-Human HER2 Protein
- Visualization Reagent
- Negative Control Reagent
- DAB Buffered Substrate
- DAB Chromogen
- Epitope Retrieval Solution (10x)
- Wash Buffer (10x) (not included in SK001)
- User-Fillable Bottles (only included in SK001)

Recommended Hematoxylin counterstain (not provided)

S3301	Hematoxylin for the Dako Autostainer
S3301	Hematoxylin for Manual Use
SK308	Mayer's Hematoxylin for Automated Link Platforms

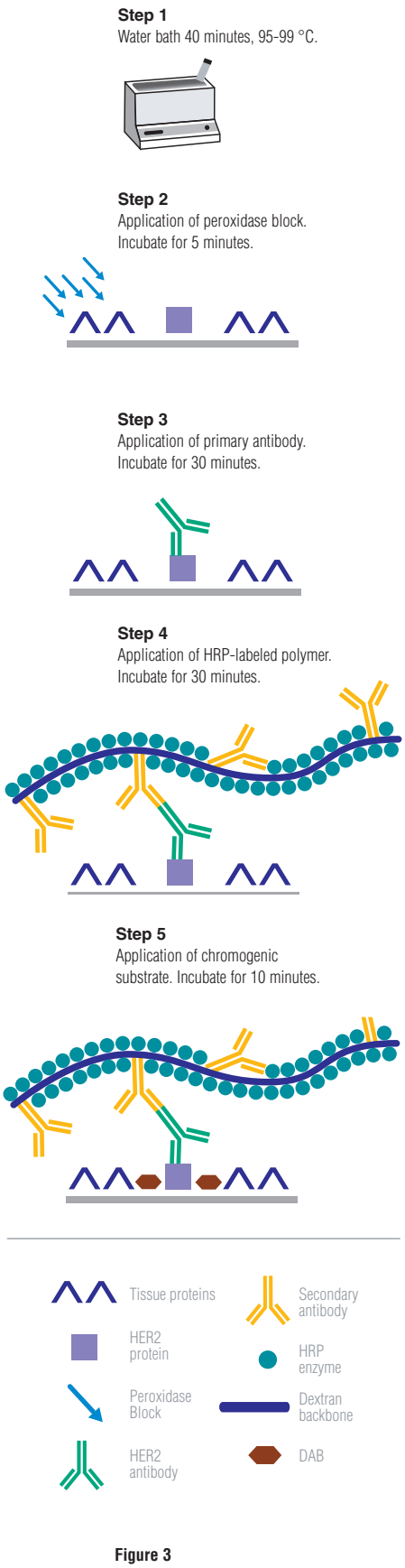


Figure 3
HercepTest™ Kit procedure

HERCEPTEST™ KIT

HER2 FISH pharmDx™ Kit

HER2 FISH pharmDx™ Kit is a fluorescence in situ hybridization assay that quantitatively determines *HER2* gene amplification in formalin-fixed, paraffin-embedded (FFPE) breast cancer tissue and FFPE specimens from patients with adenocarcinoma of the stomach, including gastroesophageal junction. The assay includes a chromosome 17 reference probe to correct for HER2 signal number in the event of chromosome 17 aneusomy.

- CEN-17 PNA probes directly labeled with fluorescein (FITC) targets the centromeric region of the chromosome (green signals)
- *HER2* DNA probe directly labeled with Texas Red fluorochrome targets the *HER2* amplicon (red signals)
- Results are expressed as a ratio of *HER2* gene copies (red signals) per number of chromosome 17 copies (green signals)

K5331	*HER2* FISH pharmDx™ Kit	20 Tests

HER2 FISH pharmDx™ Kit is a complete kit and includes

- Pre-Treatment Solution (20x)
- Pepsin, ready-to-use
- HER2/CEN-17 Probe Mix
- Stringent Wash Buffer (20x)
- Fluorescence Mounting Medium, containing DAPI
- Wash Buffer (20x)
- Coverslip Sealant

Hybridizer Instrument for Fluorescence In Situ Hybridization (FISH)

Hybridizer is a hands-free, denaturation and hybridization instrument. The system allows for semi-automation of ISH by eliminating steps in the time-intensive manual procedure.

S2450	Hybridizer	120 volt
S2451	Hybridizer	240 volt

Supporting Literature

For information about supporting literature, contact your local Dako representative or visit www.dako.com.

HercepTest™ Training Checklist

Customer Name/Institution _____

Person Trained/Title _____

Manual Staining Run ☐ Yes ☐ No If no, complete the information below.

Dako Autostainer Software Version _____ Dako Autostainer Serial Number _____

Dako Automated Link Platform Software Version _____ Dako Automated Link Platform Serial Number_____

Manual, Dako Autostainer or Automated Link Platform Procedure

	Yes	No
Control slides and kit stored at 2–8 °C?	☐	☐
Cell Line control slides and all reagents warmed to room temperature (20-25 °C) prior to starting assay?	☐	☐
Tissues fixed in 10% neutral buffered formalin?	☐	☐
Specimens air-dried at room temperature for a minimum of 12 hours (or until dry) or at 37 °C overnight or at 60 °C for one hour?	☐	☐
Specimens stained within 4-6 weeks of tissue mounted on slides when stored at room temperature?	☐	☐
Clearing solutions changed after 40 slides?	☐	☐
Deparaffinization and rehydration protocol followed?	☐	☐
Wash Buffer prepared properly? Prepare sufficient quantity of Wash Buffer by diluting Wash Buffer 10X, 1:10 in Reagent Quality Water (deionized or distilled water).	☐	☐
Distilled or deionized water (not tap water) used for water washes after last alcohol bath in deparaffinization?	☐	☐
Water bath used and set to proper temperature (95-99 °C)?	☐	☐
Epitope Retrieval Solution brought to 95 °C after slides immersed, before 40 minutes incubation started?	☐	☐
Slides allowed to cool for 20 minutes in Epitope Retrieval Solution?	☐	☐
Either alcohol or water-based hematoxylin counterstains used?	☐	☐

Manual Procedure

Distilled or deionized water (not tap water) used for water bath after Substrate-Chromogen Solution (DAB) step?	☐	☐
Diluted Wash Buffer used for all wash steps and baths (after Peroxidase-Block, Primary Antibody/Negative Control Reagent, Visualization Reagent)?	☐	☐
Buffer bath(s) changed between each step? Humid chamber used for Primary Antibody/ Negative Control Reagent/Visualization Reagent incubations?	☐	☐
Slides placed in 5 (±1) minute buffer baths between Peroxidase Block, Primary Antibody/ Negative Control Reagent, Visualization Reagent and Substrate-Chromogen Solution (DAB) steps?	☐	☐
Peroxidase-Blocking Reagent applied and specimen fully covered for five minutes?	☐	☐
Specimens fully covered with three drops (100 µL) of Primary Antibody or Negative Control Reagent for 30 minutes?	☐	☐
Visualization Reagent applied and specimen fully covered for 30 minutes?	☐	☐
Substrate-Chromogen (DAB) Solution properly prepared ? Mix 1 drop of DAB Chromogen with 1 mL DAB Buffered Substrate.	☐	☐
Substrate-Chromogen solution applied for 10 minutes and specimen fully covered?	☐	☐

Dako Autostainer or Automated Link Platform Procedure

Slides placed in buffer 5 (±1) minutes before loading onto the Dako Autostainer?	☐	☐
Appropriate protocol template used?	☐	☐
For each slide, is 200 µL of Primary Antibody or Negative Control Reagent applied?	☐	☐
Was the Dako Autostainer/Automated Link Platform program reviewed for accuracy?	☐	☐
Slides rinsed with buffer between steps and double rinsed after the Visualization Reagent step?	☐	☐
Substrate-Chromogen (DAB) Solution prepared properly? Dako Autostainer: Add 11 drops of DAB Chromogen to one vial of DAB Buffered Substrate. Automated Link Platform: Mix an appropriale amount of DAB Buffered Substrate with 25 µL DAB Chromogen per mL DAB Buffered Substrate.	☐	☐
Substrate-Chromogen Solution (DAB) applied for 10 minutes?	☐	☐

Instrumentation / Equipment

Is regular preventative maintenance performed on the Dako Autostainer/Automated Link Platform?	☐	☐
Do you have all the necessary equipment to perform the HercepTest™ assay according to protocol? If not, specify what is missing in comments below.	☐	☐
If you answered “No” to any of the above, you have deviated from protocol and should consult with your local Dako Technical Support Representative for assistance.	☐	☐

Additional observations or comments:

_____

_____

_____

TRAINING CHECKLIST

Technical Considerations for Optimal HercepTest™ Performance

Technical problems relating to the performance of HercepTest™ may arise in two areas, those involving sample collection and preparation prior to performing the test, and those involving the actual performance of the test itself. Technical problems relating to the performance of the test generally are related to procedural deviations and can be controlled and eliminated through training and, where necessary, clarification of the product instructions.

Protocol Recommendations

Pre-treatment Using Water Bath

Water Bath

Heat HercepTest™ Epitope Retrieval Solution in a calibrated water bath capable of maintaining the required temperature of 95-99 °C. For best results, fill a container suitable for holding slides with diluted Epitope Retrieval (1:10) Solution. Place container with Epitope Retrieval Solution in a water bath and bring the temperature of the water bath and the Epitope Retrieval Solution to 95-99 °C. Add the tissue sections mounted on slides to the container and bring the temperature of the Epitope Retrieval Solution back to 95 °C before starting the timer.

Incubation Time

Incubate the slides for 40 (±1) minutes in the preheated Epitope Retrieval Solution. Remove the container with the slides from the water bath, but keep them in the Epitope Retrieval Solution while allowing them to cool for 20 (±1) minutes at room temperature. After cooling, decant the Epitope Retrieval Solution and rinse in Wash Buffer. For optimal performance, soak sections in Wash Buffer for 5-20 minutes after epitope retrieval and prior to staining.

Pre-treatment Using PTLink

Preheat the diluted Epitope Retrieval Solution (1:10) in the Dako PT Link tank to 85 °C. Place the room temperature, deparaffinized sections in Autostainer racks and immerse the slides into the preheated Epitope Retrieval Solution. Let the PT Link warm up to 97 °C and incubate for 40 (±1) minutes at 97 °C. Leave the sections to cool in the PT Link until the temperature reaches 85 °C. Remove the PT Link tanks with the sections from the PT Link and leave the tanks on the table for 10 minutes with the lid off for further cooling. Prepare a jar/tank, eg. the PT Link Rinse Station, with diluted Dako Wash Buffer and soak sections for 5-20 minutes after epitope retrieval and prior to staining.

Only dedicated PT Link equipment can be used for HercepTest™. Pre-treatment using PT Link is currently validated for HercepTest™ for Automated Link Platforms.

Proper Incubations

All incubation times should be performed according to the package insert. Stay within ±1 minute of all incubation times. If staining must be interrupted, slides may be kept in Wash Buffer following incubation of the primary antibody for up to one hour at room temperature (20-25 °C).

Automated Staining

Dako recommends the use of HercepTest™ on a Dako Autostainer or Automated Link Platform. Use of HercepTest™ on alternative automated platforms has not been validated and may give erroneous results.

Wash Buffer

Dilute the recommended Wash Buffer 1:10 using distilled or deionized water. Store unused diluted solution, at 2-8 °C up to one month. Discard diluted solution, if cloudy in appearance.

Storage of Reagents

Reagents should be stored at 2-8 °C. Do not use after the expiration date stamped on the outside package.

Tissue Processing Considerations

Procedural deviations related to sample handling and processing can affect HercepTest™ results. Some of the variables that affect outcome are as follows:

- Specimens drying prior to fixation
- Type of fixative (only neutral-buffered formalin is recommended)
- Temperature, age, storage, pH of fixative
- Length of fixation, specimen size, ratio of size to fixative volume
- Length of time in alcohol after primary fixation
- Processing time, temperature, pressure and chemicals used
- Storage of paraffin blocks
- Storage of cut sections
- Section thickness

Tissue Processing Recommendations

Validated Fixatives

Neutral-Buffered Formalin

Fixation Times

Neutral-Buffered Formalin:

- 18-24 hours, surgical specimens
- 6-8 hours, biopsy specimens

Time to fixation and duration of fixation, if available, should be recorded for each sample.

Specimen Thickness

Tissue samples submitted for processing and embedding should not exceed 3-4 mm in thickness.

Processing and Embedding

After fixation, tissues are dehydrated in a series of alcohols and xylene, followed by infiltration by melted paraffin held at no more than 60 °C. Overheating of tissues during embedding or overheating of sections during drying can induce detrimental effects on immunostaining and, therefore, should be avoided.

The slides required for HER2 protein evaluation and tumor presence should be prepared at the same time. To preserve antigenicity, tissue sections, mounted on slides, should be stained within four to six weeks of sectioning when held at room temperature, 20-25 °C. Tissue sections should be cut into a thickness of 4-5 µm.

Review of HercepTest™ Scoring Guidelines

HercepTest™ is a semi-quantitative immunohistochemical assay to determine HER2 protein overexpression in breast cancer tissue routinely processed for histological evaluation and in formalin-fixed, paraffin-embedded cancer tissues from patients with adenocarcinoma of the stomach, including gastroesophageal junction.

For the determination of HER2 protein overexpression, only the membrane staining intensity and pattern should be evaluated using the scale presented on page 12. Slide evaluation should be performed using a bright field microscope.

Validation of the Assay

Included in each HercepTest™ kit are control slides representing different levels of HER2 protein expression: MDA-231(0), MDA-175 (1+) and SK-BR-3 (3+). The first step of interpretation is to evaluate the control cell lines. The control cell lines have been provided for qualifying the procedure and reagents, not as an interpretation reference. No staining of the 0 control cell line, MDA-231, partial brown membrane rimming in the 1+ control cell line, MDA-175, (refer to the Interpretation Guide for 1+ Cell Line on the next page), and presence of complete intense brown membrane staining (rimming) in the 3+ control cell line, SK-BR-3, indicates a valid assay. If any of the control cell lines perform outside of these criteria, all results with the patient specimens should be considered invalid.

Next, the positive tissue control slide known to contain the HER2 antigen, stained with HercepTest™ and fixed and processed similarly to the patient slides, should be evaluated for indication of correctly prepared tissue and proper staining technique. The ideal positive tissue control is weakly positive. The presence of a brown reaction product at the cell membrane is indicative of positive reactivity. Verify that the negative tissue control slide from the same staining run demonstrates no reactivity.

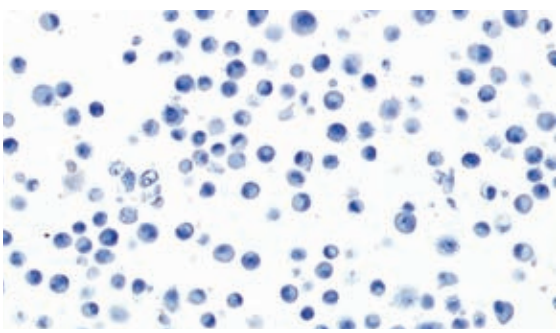


Figure 4
0 control cell line, MDA-231, stained with HercepTest™. No staining of the membrane is observed.
20x magnification.

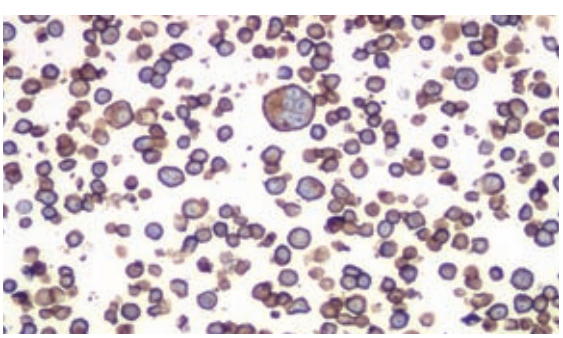


Figure 5
3+ control cell line, SK-BR-3, stained with HercepTest™. A strong staining of the entire membrane is observed.
20x magnification.

GUIDELINES

Interpretation Guide for 1+ Cell Line

The 1+ control cell line can display different categories of HER2-specific cellular staining. Cells displaying a partial brown membrane rimming, where the immunostaining is punctate and discontinuous (Figure 6, 1a) are the true indicators of a valid staining run. In some cells, the partial brown membrane rimming is more borderline (but still considered positive) consisting of a punctate and discontinuous immunostaining of both membrane and cytoplasm (Figure 6, 1b). The borderline cells depicted here may reflect the difference in quality between images and true microscopy. In a normal IHC staining run of the 1+ control cell line, few cells will display a circumferential brown cell membrane staining (Figure 6, 2). In addition, in some cells dot-like immunostaining can be observed in the Golgi region of the cytoplasm (Figure 6, 3).

The different categories of HER2-specific cellular staining may be reflected in the different appearances of acceptable 1+ cellular staining runs, e.g. low (Figure 7) and moderate (Figure 8).

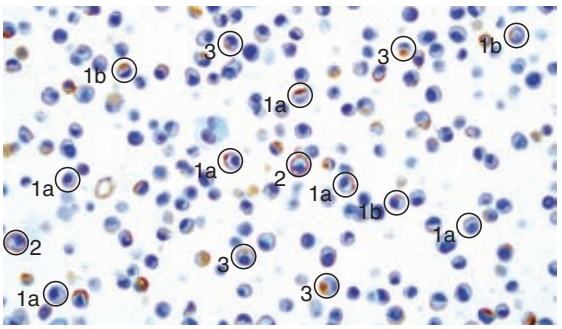


Figure 6
The 1+ control cell line, MDA-175 (20x), may display different categories of HER2-specific cellular stainings. Only the HER2 specific staining displayed as a partial brown membrane rimming – is used to validate the staining run.
Note: The image only represents approximately 50% of a 20x microscope visual field.

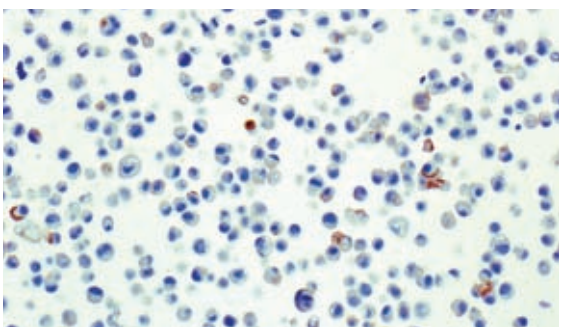


Figure 7
1+ control cell line, MDA-175 (20x), acceptable staining run with punctate and discontinuous membrane staining in a small number of cells. The "low-limit appearance" may reflect the difference in quality between images and true microscopy.
Note: The image only represents approximately 50% of a 20x microscope visual field.

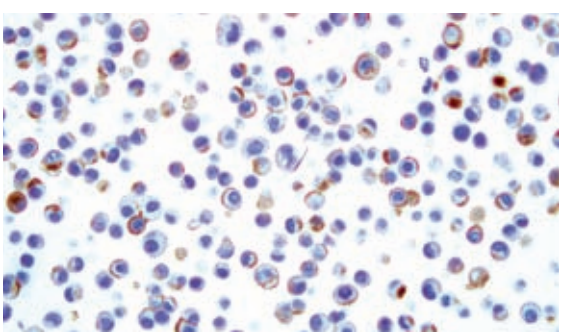


Figure 8
1+ control cell line, MDA-175 (20x), acceptable staining run with punctate and discontinuous membrane staining in a moderate number of cells.
Note The image only represents approximately 50% of a 20x microscope visual field.

Guidelines for Scoring

Use of the attached scoring system has proved reproducible both within and among laboratories. Dako recommends that scoring should always be performed within the context of the pathologist's past experience and best judgment in interpreting IHC stains. Only specimens from patients with stomach or gastroesophageal junction adenocarcinoma should be scored. In cases with intestinal metaplasia and gastric adenocarcinoma in the same specimen, only the gastric adenocarcinoma component should be scored. Figure 9 on the next page shows examples of staining patterns.

Surgical Specimens

Score to Report	HER2 Protein Overexpression Assessment	Staining Pattern
0	Negative	No reactivity or membranous reactivity in < 10% of tumor cell
1+	Negative	Faint/barely perceptible membranous reactivity in ≥ 10% of tumor cells. Cells are reactive only in part of their membrane
2+	Equivocal	Weak to moderate complete, basolateral or lateral membranous reactivity in ≥ 10% of tumor cells
3+	Positive	Strong complete, basolateral or lateral membranous reactivity in ≥ 10% of tumor cells

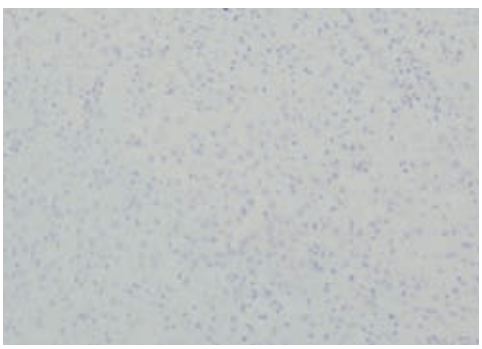
Guidelines based on Hofmann M, Stoss O, Shi D, Büttner R, van de Vijver M, Kim W, et al. Assessment of a HER2 scoring system for gastric cancer: results from a validation study. Histopath 2008; 52:797–805.

Biopsy Specimens

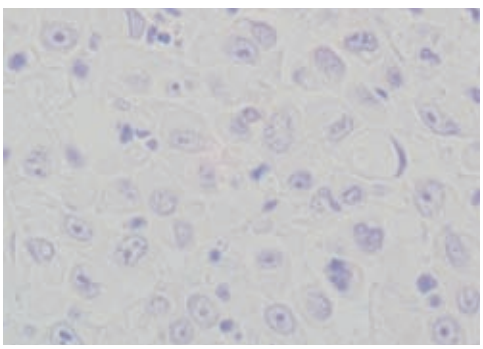
Score to Report	HER2 Protein Overexpression Assessment	Staining Pattern
0	Negative	No reactivity or no membranous reactivity in any tumor cell
1+	Negative	Tumor cell cluster with a faint/barely perceptible membranous reactivity irrespective of percentage of tumor cells stained
2+	Equivocal	Tumor cell cluster with a weak to moderate complete, basolateral or lateral membranous reactivity irrespective of percentage of tumor cells stained
3+	Positive	Tumor cell cluster with a strong complete, basolateral or lateral membranous reactivity irrespective of percentage of tumor cells stained

Guidelines based on Hofmann M, Stoss O, Shi D, Büttner R, van de Vijver M, Kim W, et al. Assessment of a HER2 scoring system for gastric cancer: results from a validation study. Histopath 2008; 52:797–805.

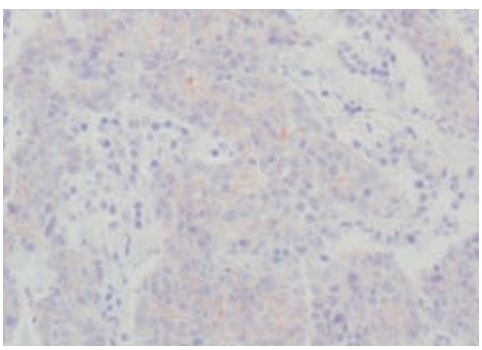
For interpretation of HercepTest™ - stained biopsies, a cluster of at least five tumor cells is recommended.



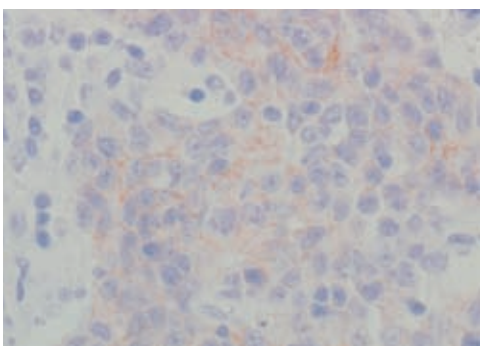
Score: **0** (20x)



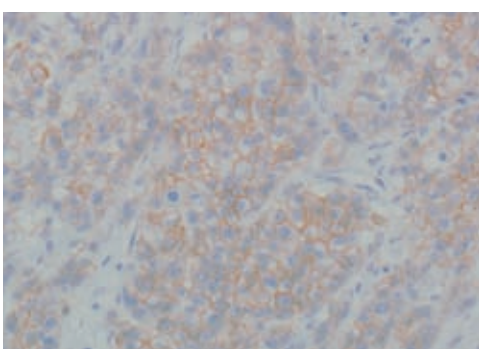
Score: **0** (40x)



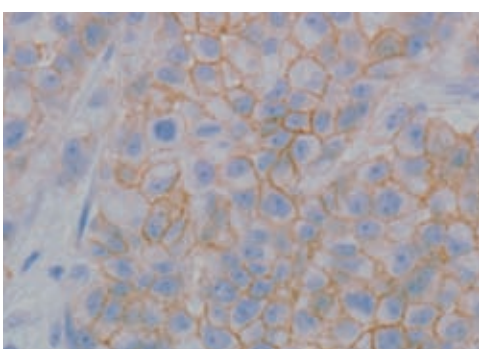
Score: **1+** (20x)



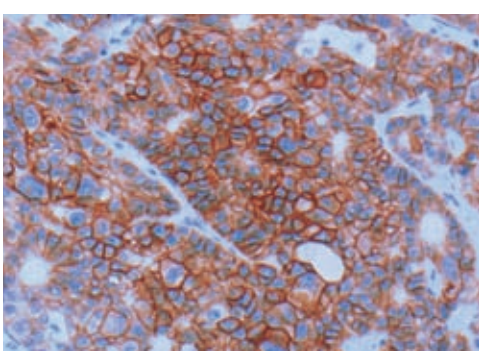
Score: **1+** (40x)



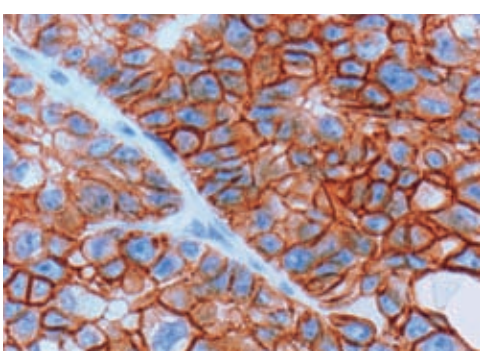
Score: **2+** (20x)



Score: **2+** (40x)



Score: **3+** (20x)



Score: **3+** (40x)

Figure 9
Examples of staining patterns for tissue scored 0,1+, 2+ and 3+ at both 20x and 40x magnification.

Complete, Basolateral and Lateral HER2 Staining

Incomplete HER2 membrane staining (basolateral and lateral) is common in gastric tissue, and is caused by glandular formations. A basolateral staining is a staining without luminal staining (making the membrane appear ”U”shaped). A lateral membrane staining is a staining without luminal and basal staining (making the membrane appear ”II” shaped) (Figure 10).

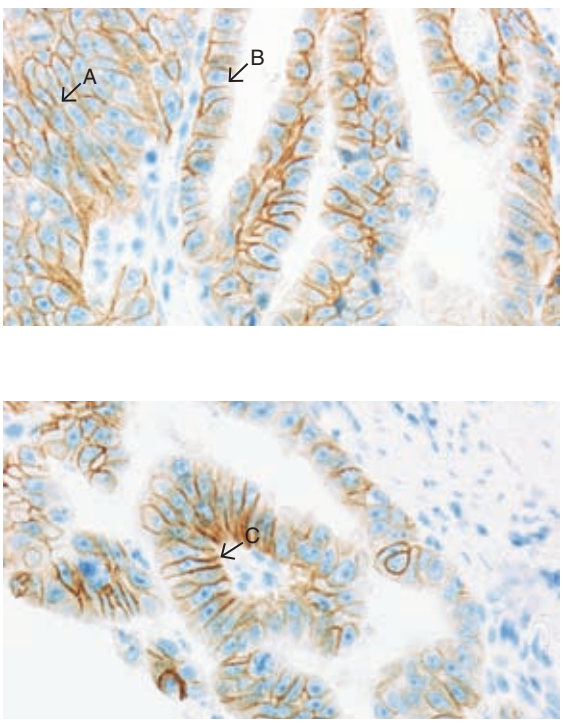


Figure 10
Gastric cancer. Score 3+. HercepTest™ stains showing complete (A), basolateral (B) and lateral (C) HER2 membrane staining. 40x magnification.

Cut-off Numbers

Due to a high degree of heterogeneity in gastric cancer tissue the cut-off number for HER2 positive stained tumor cells is different for surgical and biopsy specimens. The cut-off for surgical specimens is 10% of positive stained tumor cells (Figure 11) and for biopsy specimens it is a cluster of at least 5 positive stained tumor cells (Figure 12).

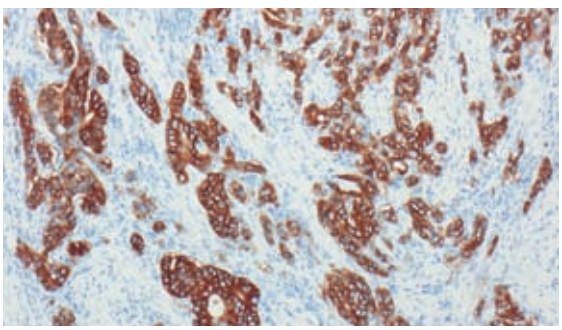


Figure 11
Gastric cancer, surgical specimen. Score 3+.10% or more tumor cells exhibit complete, basolateral or lateral membrane staining. HER2 staining intensity is strong. 20x magnification.

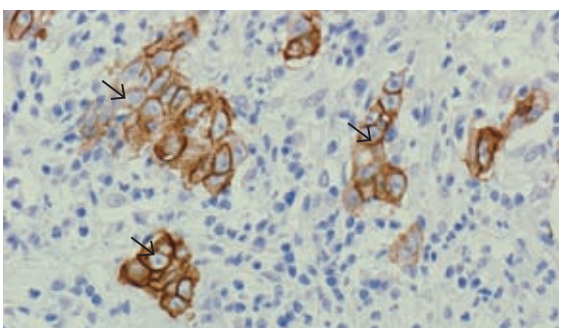


Figure 12
Gastric cancer, biopsy specimen. Score 3. Example of clusters of at least 5 HER2 stained tumor cells (arrows). HER2 staining intensity is strong. 40x magnification.

Recommendation for Interpretation of HercepTest™ - Gastric Cancer

Dako emphasizes that scoring of HercepTest™ must be performed in accordance with the guidelines establised in the package insert and within the context of best practices and the pathologist's experience and best medical judgment. This manual will highlight areas of interpretation potentially problematic for HercepTest™ users.

Steps for HercepTest™ interpretation

1. Evaluate the Control Cell Lines to validate the assay performance.

2. Evaluate the Positive and Negative Control Slides.

3. A hematoxylin and eosin (H&E) stained section of the tissue specimen is recommended for the first evaluation. The tumor may not be obvious when looking at the sample stained with HercepTest™. An H&E stained slide allows the pathologist to verify the presence of the tumor. HercepTest™ should be performed on a paired section (serial section) from the same paraffin block of the specimen.

4. Evaluate the sections stained for HER2 protein at low power magnification first. The majority of positive cases will be obvious at low power magnification.

5. For 1+ cases, use 40x objective magnification to verify membrane staining.

6. For 2+ cases, use 10x-20x objective magnification to verify membrane staining.

Surgical specimen

1. Well-preserved and well-stained areas of the section should be used to make a determination of the percent of positive stained tumor cells.

2. If a majority of tumor cells demonstrate complete, basolateral or lateral membrane staining, the staining is either 2+ or 3+.

3. If there is complete, basolateral or lateral membrane staining at a strong intensity in equal to or more than 10% of the tumor cells in surgical specimens, the score of the specimen is 3+.

4. If there is complete, basolateral or lateral membrane staining at a weak to moderate intensity in equal to or more than 10% of the tumor cells in surgical specimens, the score of the specimen is 2+.

5. If equal to or more than 10% of the tumor cells in surgical specimens, stained only in part of their membrane, have a faint/barely perceptible intensity, the score of the specimen is 1+.

6. If no staining is observed the score of the surgical specimen is 0.

7. If less than 10% of the tumor cells in surgical specimens have staining, irrespective of the staining pattern (e.g. complete, basolateral, lateral or part of their membrane), the score is 0.

Biopsy specimen

1. If there is a tumor cell cluster of at least 5 stained tumor cells with a strong complete, basolateral or lateral membrane staining, the score of the biopsy specimen is 3+, irrespective of percentage of tumor cells stained.

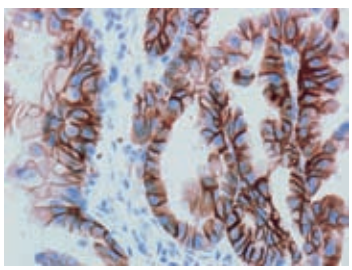
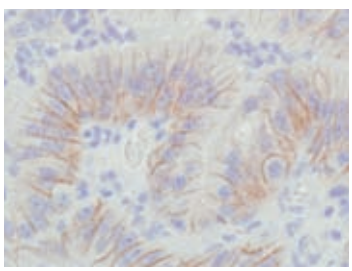
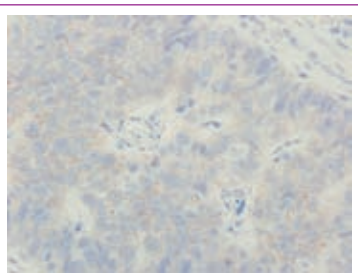
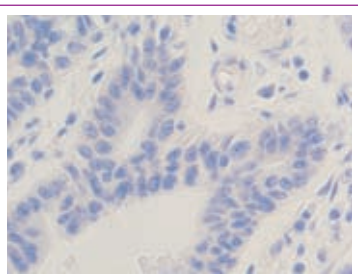
2. If there is a tumor cell cluster of at least 5 stained tumor cells with a weak to moderate complete, basolateral or lateral membrane staining, the score of the biopsy specimen is 2+, irrespective of percentage of tumor cells stained.

3. If there is a tumor cell cluster of at least 5 stained tumor cells with a faint/barely perceptible membrane staining and cells are stained only in part of their membrane, the score of the biopsy specimen is 1+, irrespective of percentage of tumor cells stained.

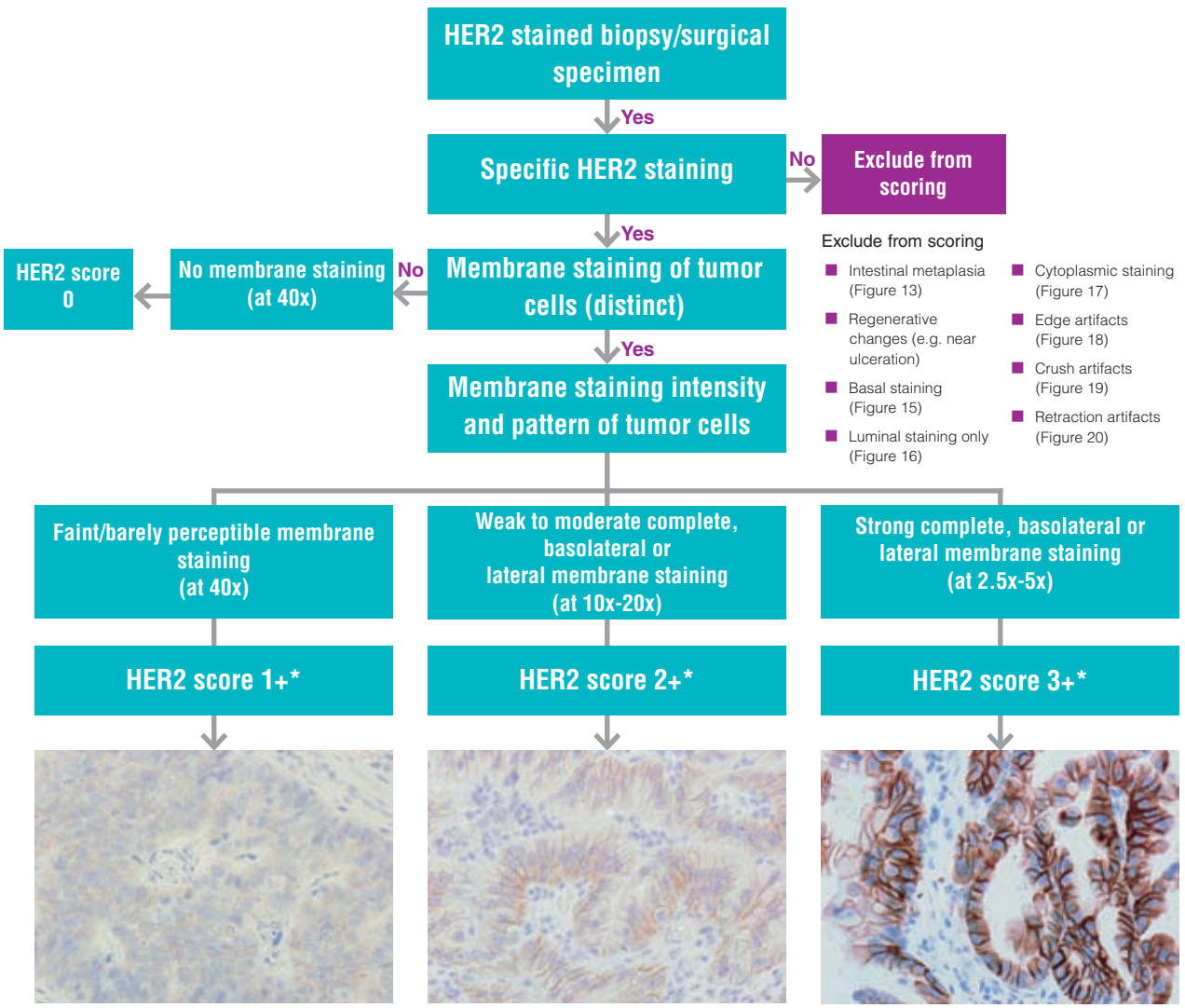
4. If no staining is observed the score of the biopsy specimen is 0.

5. If membrane staining (irrespective of staining intensity) is observed in less than 5 clustered tumor cells, the score of the biopsy specimen is 0.

Magnification and HER2 Score

HER2 Score		Membrane Staining Intensity	Magnification
3+		Strong	Use **2.5x-5x** objective magnification to verify membrane staining
2+		Weak to moderate	Use **10x-20x** objective magnification to verify membrane staining
1+		Faint/barely visible	Use **40x** objective magnification to verify membrane staining
0		No membrane staining	Use **40x** objective magnification to evaluate specimen

Step-by-Step Evaluation of HercepTest™ Stained Gastric Cancer Specimens



* Must at least be a cluster of 5 stained tumor cells for biopsy specimens and at least 10% stained tumor cells for surgical specimens.

Exclude From Scoring

Intestinal metaplasia

For determination of HER2 protein expression only specimens from patients with adenocarcinoma of the stomach, including gastroesophageal junction, should be scored. In cases with the occurrence of intestinal metaplasia and gastric adenocarcinoma in the same specimen, only the gastric adenocarcinoma component should be scored (Figure 13 and Figure 14). Regenerative changes (e.g. near ulceration) should be excluded from scoring.

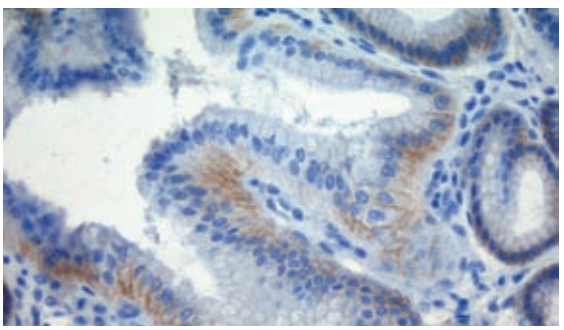


Figure 13
Gastric cancer specimen demonstrating foveolar and intestinal metaplasia staining. Exclude intestinal metaplasia from scoring. Courtesy of Targos Molecular Pathology GmbH.

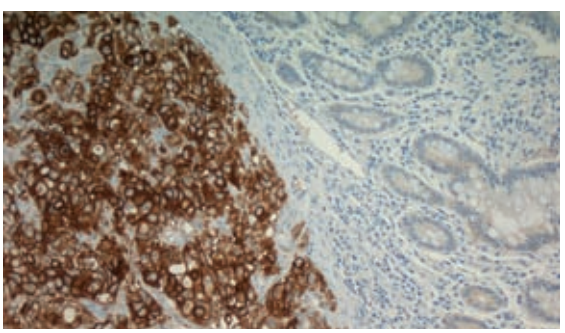


Figure 14
Gastric cancer. Score 3+. Intestinal metaplasia (right) and gastric cancer (left) in the same specimen. Exclude intestinal metaplasia from scoring. Courtesy of Targos Molecular Pathology GmbH.

EXCLUDE FROM SCORING

Basal staining only and luminal staining only

For determination of HER2 protein expression, only the membrane staining intensity and pattern should be evaluated. Complete, basolateral and lateral membrane staining pattern should be evaluated whereas basal staining only (Figure 15) and luminal staining only (Figure 16) are excluded from scoring.

Cytoplasmic staining

Cytoplasmic staining (Figure 17) should be considered non-specific staining and is not to scored.

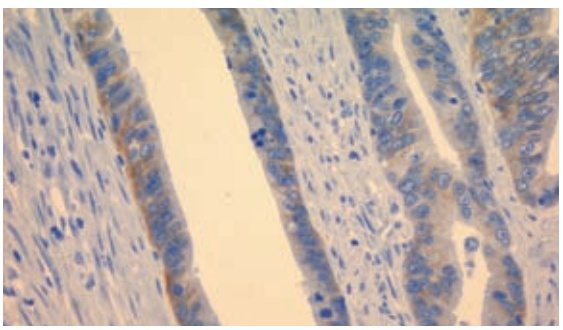


Figure 15
Gastric cancer. Demonstration of basal staining only. To be excluded from scoring. Courtesy of Targos Molecular Pathology GmbH.

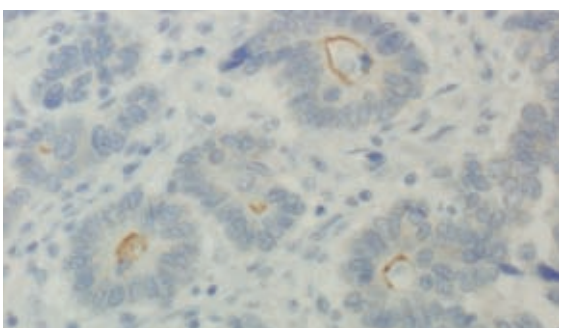


Figure 16
Gastric cancer. Demonstration of luminal staining only. To be excluded from scoring.

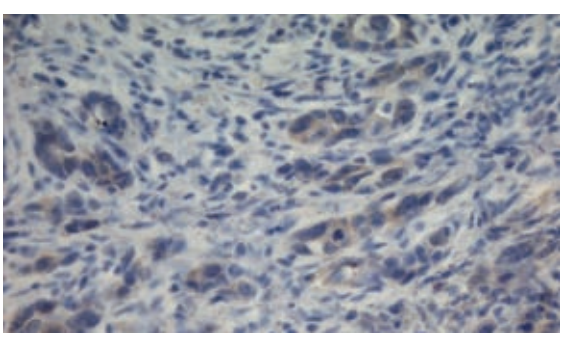


Figure 17
Gastric cancer. Demonstration of non-specific cytoplasmic staining. To be excluded from scoring. Courtesy of Targos Molecular Pathology GmbH.

Edge artifacts

Edge artifacts are usually linked to the pre-analytic handling of the tissue. Often the method of surgical extration is the cause (see Crush artifacts on the next page). This phenomenon is more frequently observed for stereotactic needle biopsies.

Increased staining intensity is frequently observed around the periphery of the tissue section, known as ”the edge effect”.

- The edge effect represents artifacts due to tissue drying prior to fixation.
- If staining is only observed at the edge of the tissue section, scoring of the tissue specimen should be avoided (Figure 18).

Inadequate fixation of tissue samples rendering the central portion of the tissue sub-optimal fixed relative to the peripheral areas, may mimic edge artifact. In these circumstances, the immunoreactivity in the sub-optimal central portion may be mistakenly interpreted as false-negative as compared to the correct immunoreactivity observed at the section periphery which has optimal fixation.

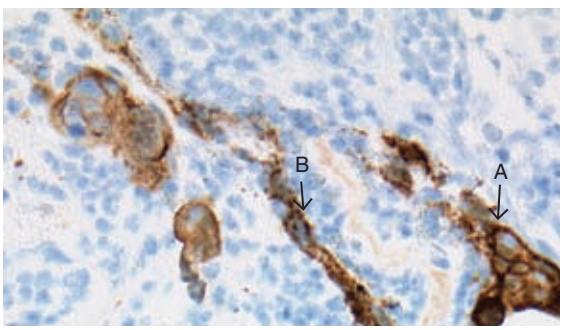


Figure 18
Gastric cancer showing edge artifacts (A) and granular unspecific cytoplasmic staining (B). Courtesy of Targos Molecular Pathology GmbH.

EXCLUDE FROM SCORING

EXCLUDE FROM SCORING

Crush artifacts

Crush artifacts are related to edge artifacts. This artifact may be encountered more often in needle biopsies. It is presumed that the tissue injury occurs during the extraction of the tissue from the needle rather than from the actual biopsy process. Regardless, the compression of the tissue along the edges of the needle core can produce a linear staining that should be interpreted as an artifact.

Tissue areas with crushed cells typically demonstrate condensed nuclei and should be avoided in scoring. Deposition of the chromogen is characteristic in areas where the cells are crushed, while well-preserved cells are devoid of immunoreactivity (Figure 19).

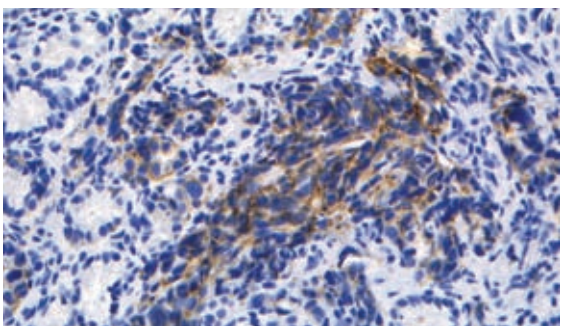


Figure 19
Gastric cancer biopsy specimen showing crush artifacts. Courtesy of Targos Molecular Pathology GmbH

Retraction artifacts (artifacts on a cellular level)

Retraction artifacts are small spaces in the tissue where antibody and chomogen can pool forming circumferential depositions. Retraction of epithelial cells from stroma may create small spaces where the reagent pool around the epithelial cells forms a circumferential deposition of the brown end product (Figure 20). This artifact requires thorough examination of the intercellular areas (i.e. cell-to-cell interface not the cell-to-stroma interface).

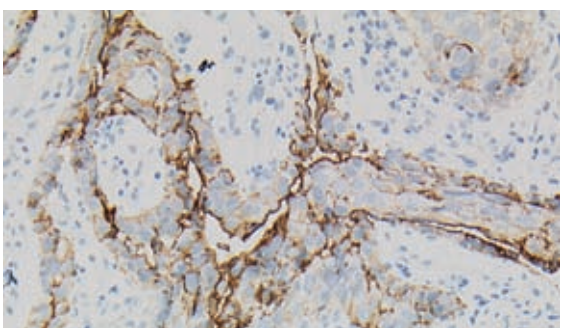


Figure 20
Gastric cancer showing retraction artifacts. Moderate staining intensity in the cell-to-cell interface. Score 2+. Courtesy of Targos Molecular Pathology GmbH.

Background Staining

Background Staining

Background staining is defined as diffuse, non-specific staining of a specimen. It is caused by several factors. These factors include, but are not limited to: pre-analytic fixation and processing of the specimen, incomplete removal of paraffin from sections, and incomplete rinsing of slides.

The use of fixatives other than neutral buffered formalin may be a source to background staining. Background staining with HercepTest™ is rare.

Possible Causes of Background Staining

- Improper drying of slides
- Improper deparaffinization procedure
- Use of different buffer than recommended
- Incomplete rinsing of reagents from slides

Evaluating the non-specific background staining of the negative test specimen is useful in interpreting the level of background staining in the positive test specimen. If background staining is significant, the specific staining must be interpreted with caution.

Heterogenous Staining

Heterogenous staining pattern is often observed in gastric cancer tissue (Figure 21, 22, 23) due to true biological difference in HER2 protein expression levels.

- The pathologist's experience and judgment is important in the evaluation of heterogeneous staining.
- Review these cases at low power magnification.
- **For surgical specimens:** There must be at least 10% or more tumor cells demonstrating complete, basolateral or lateral membrane staining for the score to be at least 2+ or greater.
- **For biopsy specimens:** There must be at least 5 clustered tumor cells demonstrating complete, basolateral or lateral membrane staining for the score to be at least 2+ or greater.

If there is any doubt about the cause of heterogeneity (e.g. artifacts), confirmation by FISH is recommended.

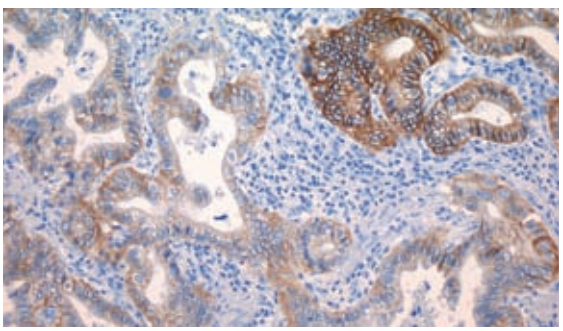


Figure 21
Gastric cancer with example of heterogeneous staining. Characteristic feature: 2+ score on the left and 3+ on the right. 10% or more of the tumor cells demonstrate strong, complete, basolateral or lateral membrane staining. Score of the gastric cancer specimen is 3+. Courtesy of Targos Molecular Pathology GmbH.

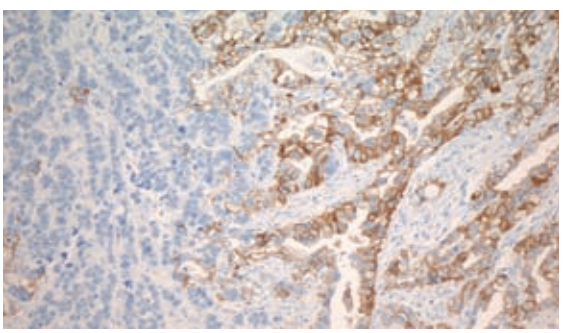


Figure 22
Gastric cancer with example of heterogeneous staining. Characteristic feature: 0 score on the left and 3+ on the right. 10% or more of the tumor cells demonstrate strong complete, basolateral or lateral membrane staining. Score of the gastric cancer specimen is 3+. Courtesy of Targos Molecular Pathology GmbH.

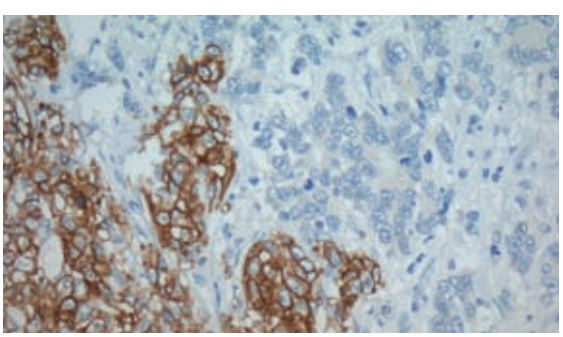


Figure 23
Gastric cancer with example of heterogeneous staining. Characteristic feature: 0 score on the right and 3+ on the left. 10% or more of the tumor cells demonstrate strong complete, basolateral or lateral membrane staining. Score of the gastric cancer specimen is 3+. Courtesy of Targos Molecular Pathology GmbH.

Homogenous Staining

In a gastric cancer tissue specimen with a homogenous HER2 staining pattern, the individual tumor cells display almost uniform immunostaining (Figure 24, 25, 26).

- **For surgical specimens:** There must be at least 10% or more of the tumor cells demonstrating complete, basolateral or lateral membrane staining for the score to be at least 2+ or greater.
- **For biopsy specimens:** There must be at least 5 clustered tumor cells demonstrating complete, basolateral or lateral membrane staining for the score to be at least 2+ or greater.

Interpretation of homogeneous staining should be based on an overall evaluation of all tumor cells.

Review of the average staining in the whole section should be performed.

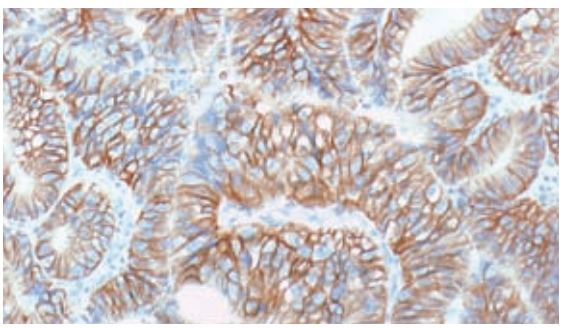


Figure 24
Gastric cancer with 3+ homogeneous staining.
20x magnification.

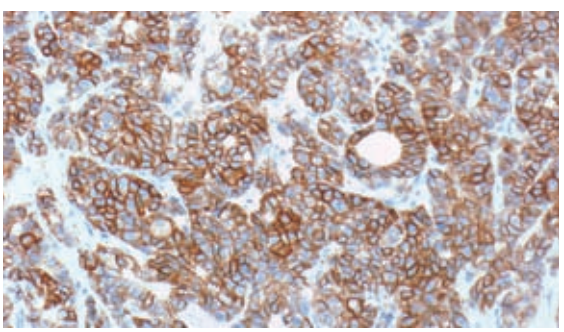


Figure 25
Gastric cancer with 3+ homogeneous staining.
20x magnification.

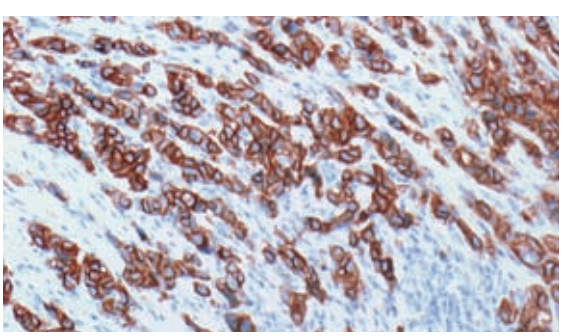


Figure 26
Gastric cancer with 3+ homogeneous staining.
20x magnification.

HER2 Expression in Gastric Cancer

Adenocarcinoma of the stomach including gastroesophageal junction (GEJ) is also referred to as gastric cancer in this document.

Figure 27-74 show examples of staining patterns

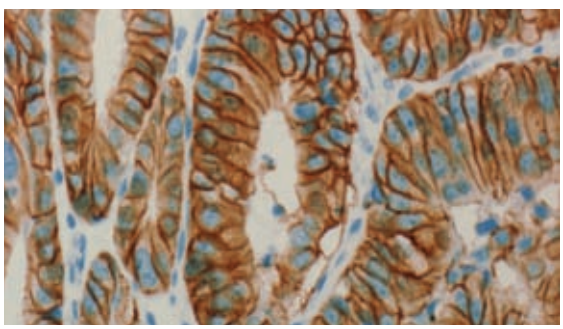


Figure 27
Adenocarcinoma of the stomach. Score 3+. 40x magnification

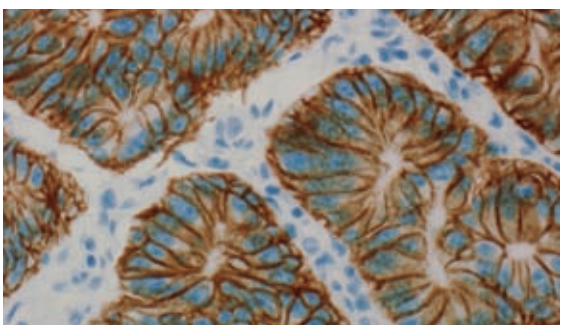


Figure 30
Adenocarcinoma of GEJ. Score 3+. 40x magnification

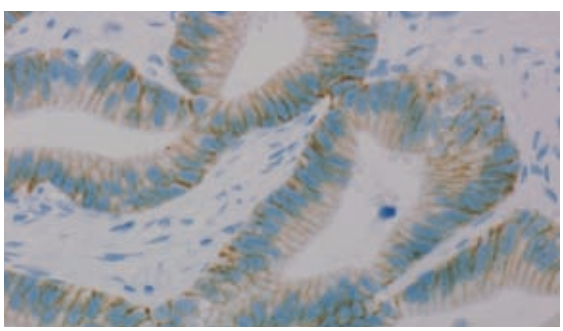


Figure 28
Adenocarcinoma of the stomach. Score 2+. 40x magnification

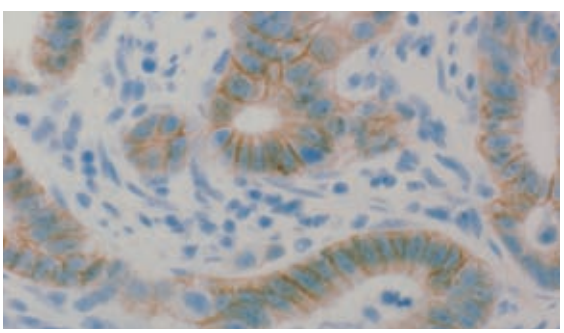


Figure 31
Adenocarcinoma of GEJ. Score 2+. 40x magnification

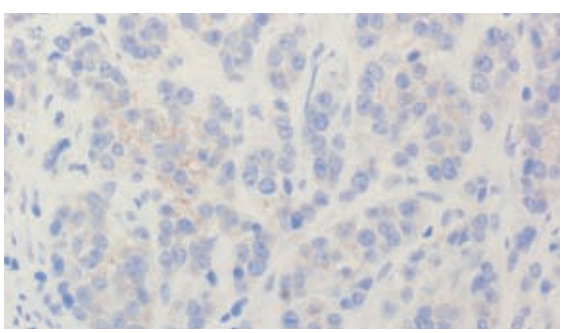


Figure 29
Adenocarcinoma of the stomach. Score 1+. 40x magnification

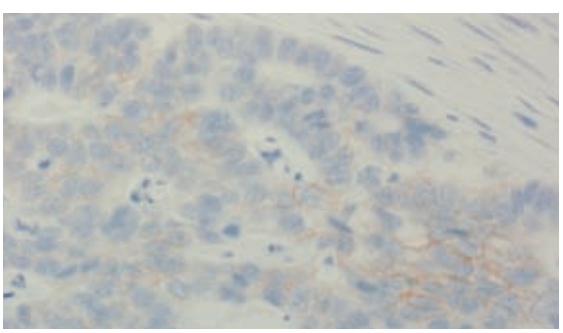


Figure 32
Adenocarcinoma of GEJ. Score 1+. 40x magnification

Staining Images

HercepTest™ Score 0

No staining is seen in this gastric cancer.

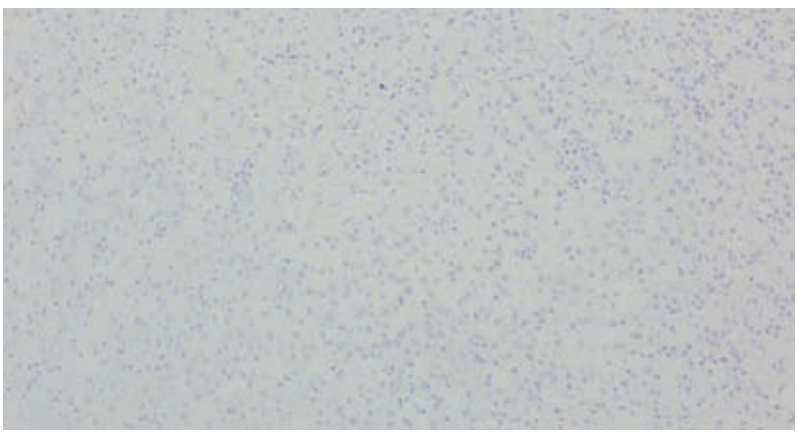


Figure 33
Gastric cancer. Score 0
10x magnification.

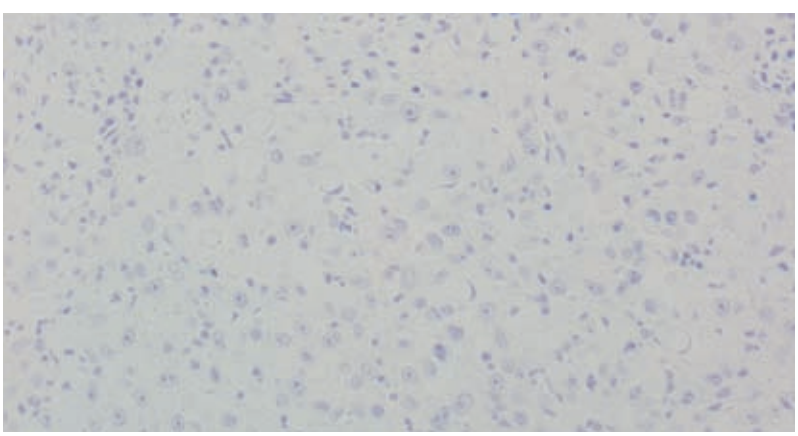


Figure 34
Gastric cancer. Score 0
20x magnification.

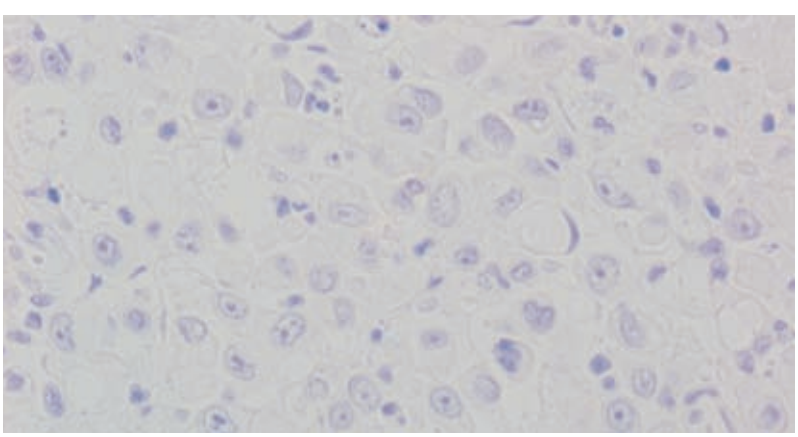


Figure 35
Gastric cancer. Score 0
40x magnification.

HercepTest™ Score 1+

The tumor cells are weakly stained. Tumor cells are stained only in part of their membrane.

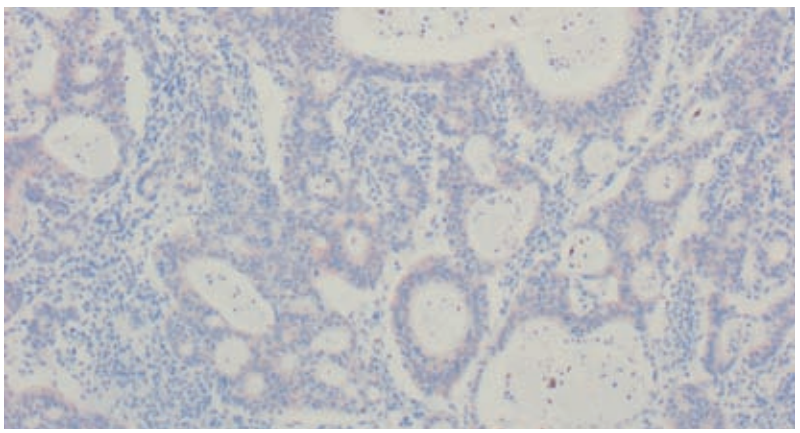


Figure 36
Gastric cancer. Score 1+
10x magnification.

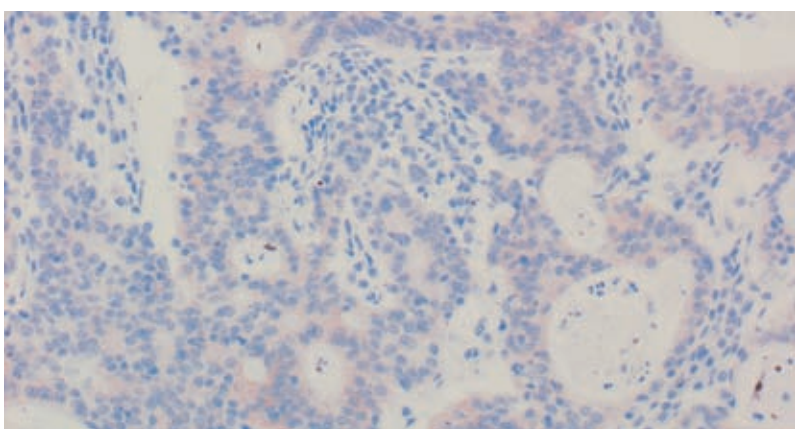


Figure 37
Gastric cancer. Score 1+
20x magnification.

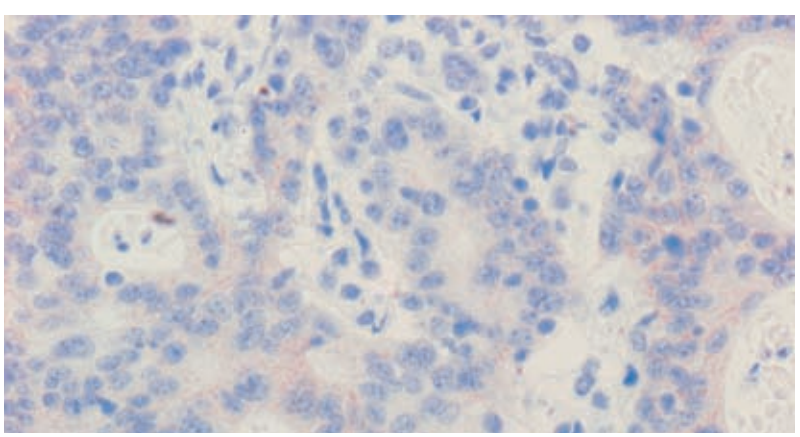


Figure 38
Gastric cancer. Score 1+
40x magnification.

HercepTest™ Score 1+

The tumor cells are weakly stained. Tumor cells are stained only in part of their membrane.

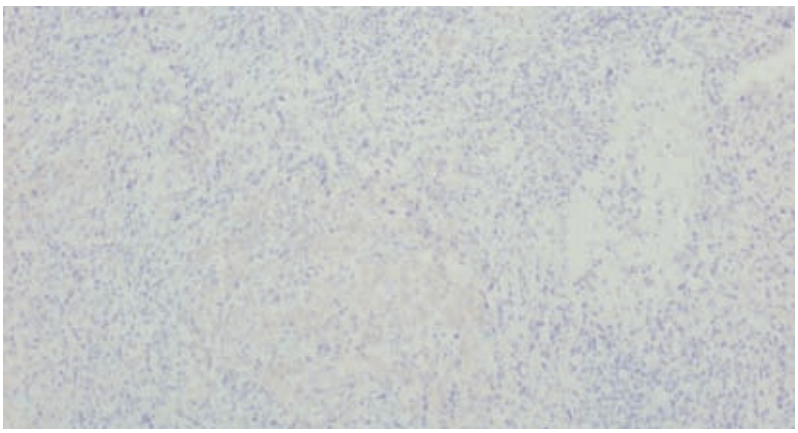


Figure 39
Gastric cancer. Score 1+
10x magnification.

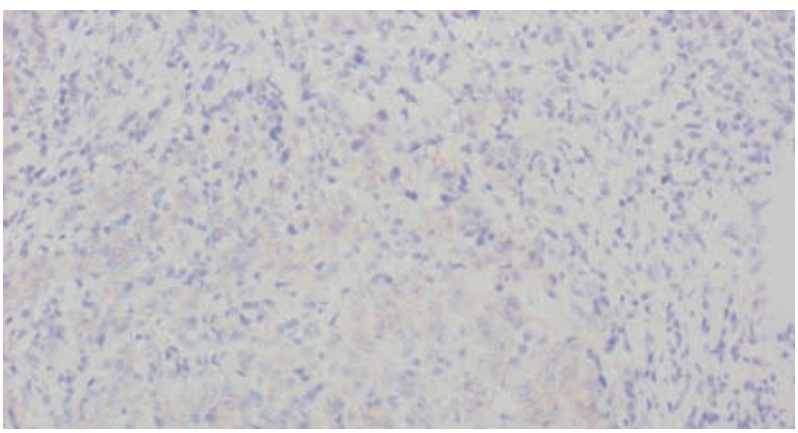


Figure 40
Gastric cancer. Score 1+
20x magnification.

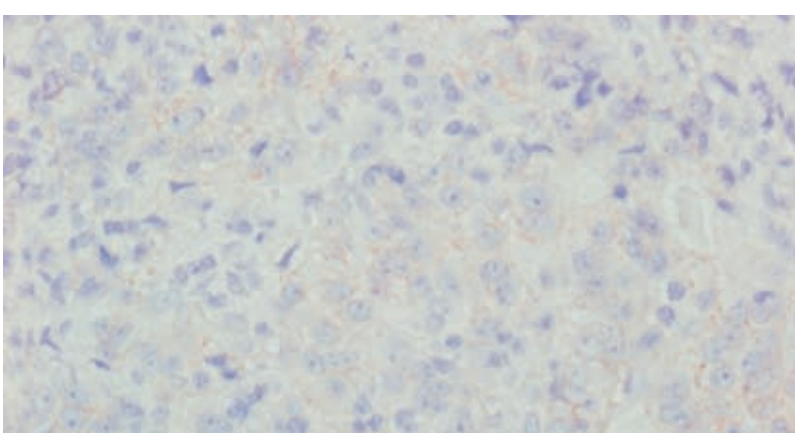


Figure 41
Gastric cancer. Score 1+
40x magnification.

HercepTest™ Score 1+

The tumor cells are weakly stained. Tumor cells are stained only in part of their membrane.

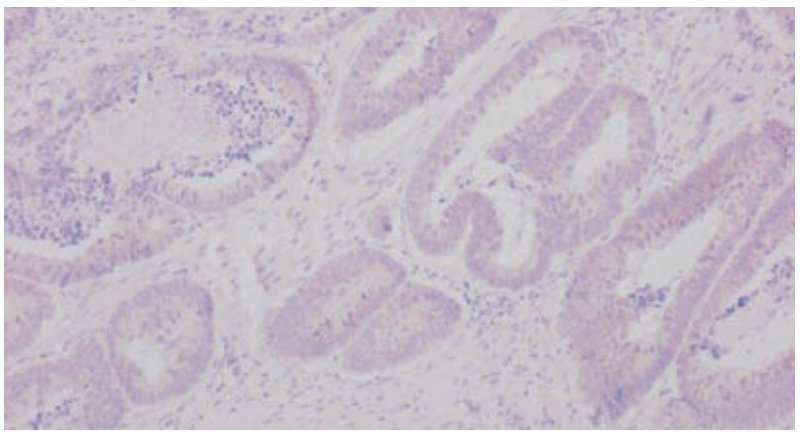


Figure 42
Gastric cancer. Score 1+
10x magnification.

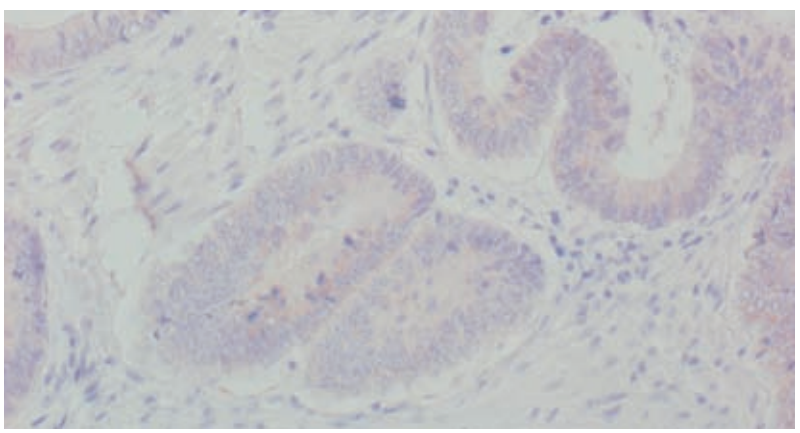


Figure 43
Gastric cancer. Score 1+
20x magnification.

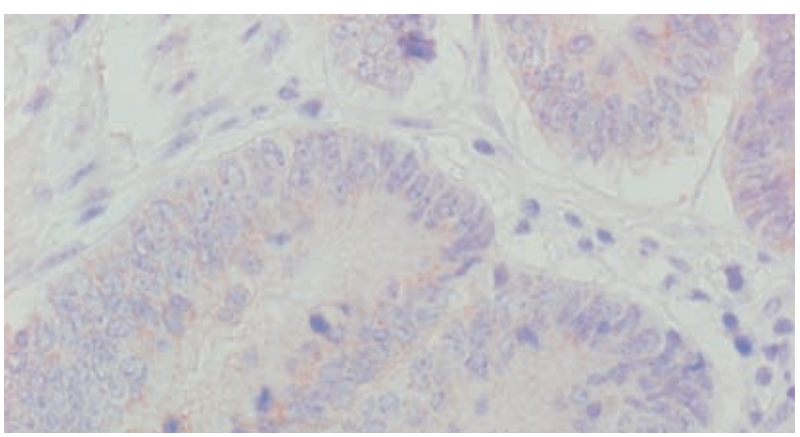


Figure 44
Gastric cancer. Score 1+
40x magnification.

HercepTest™ Score 1+

The tumor cells are weakly stained. Tumor cells are stained only in part of their membrane.

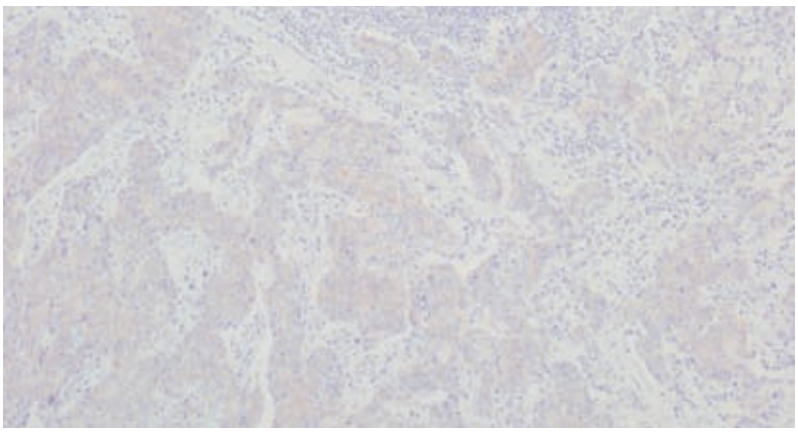


Figure 45
Gastric cancer. Score 1+
10x magnification.

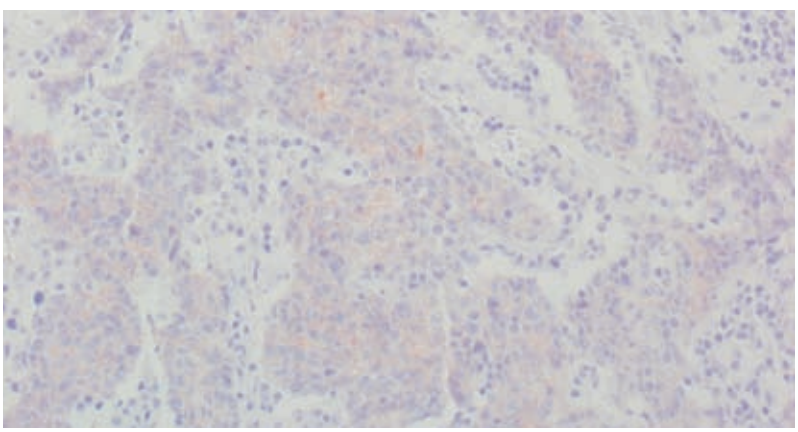


Figure 46
Gastric cancer. Score 1+
20x magnification.

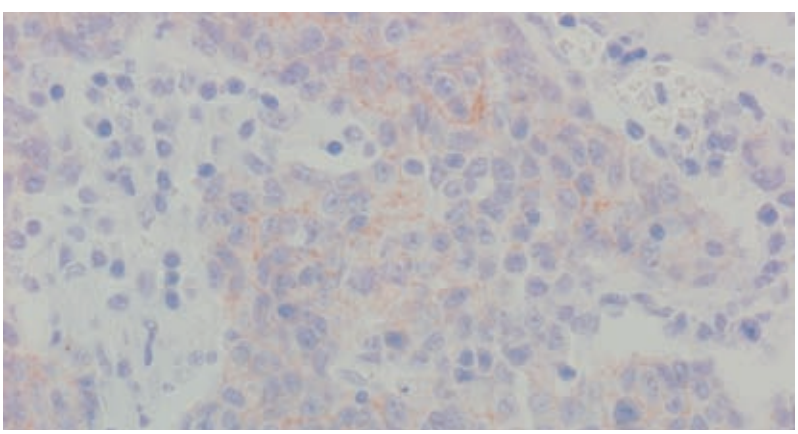


Figure 47
Gastric cancer. Score 1+
40x magnification.

HercepTest™ Score 2+

The tumor cells exhibit complete, basolateral or lateral membrane staining; the intensity is weak to moderate.

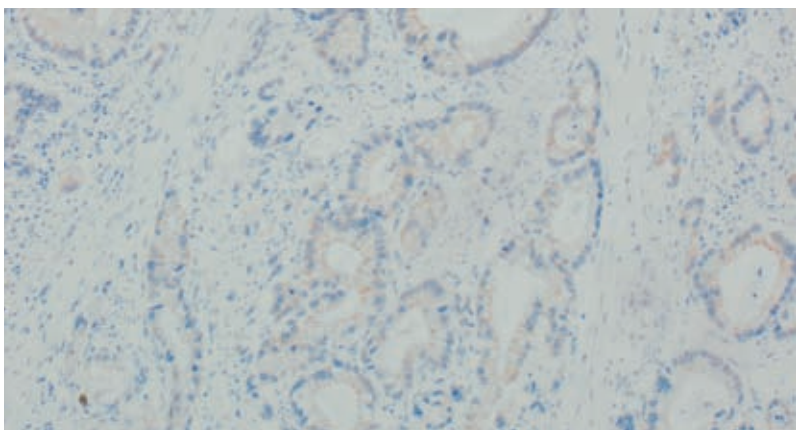


Figure 48
Gastric cancer. Score 2+, 10x magnification.

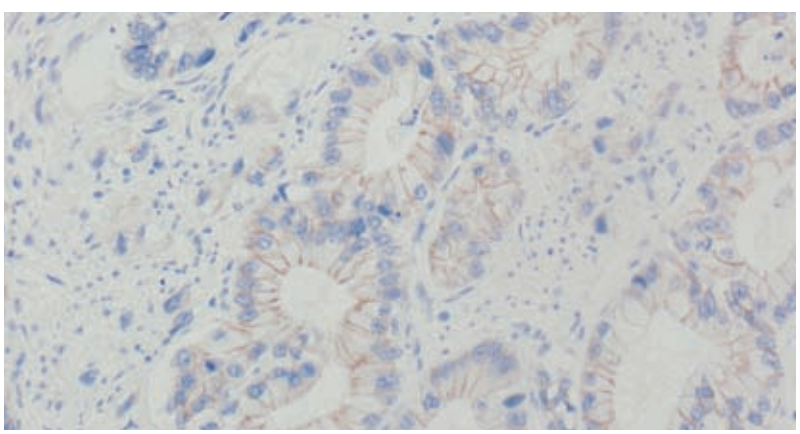


Figure 49
Gastric cancer. Score 2+, 20x magnification.

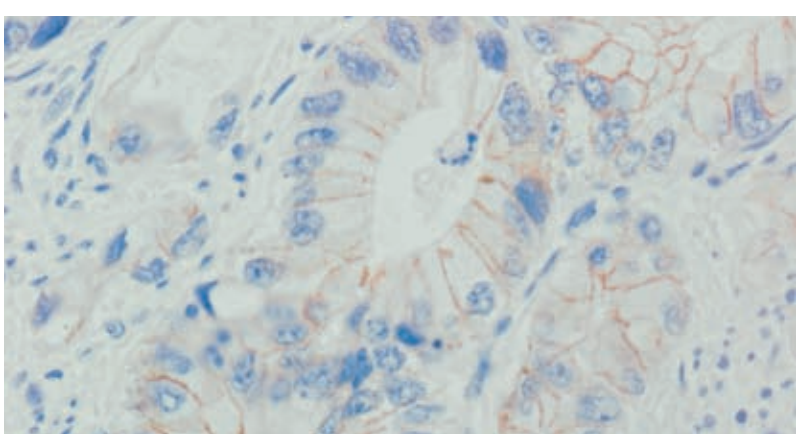


Figure 50
Gastric cancer. Score 2+, 40x magnification.

HercepTest™ Score 2+

The tumor cells exhibit complete, basolateral or lateral membrane staining; the intensity is weak to moderate.

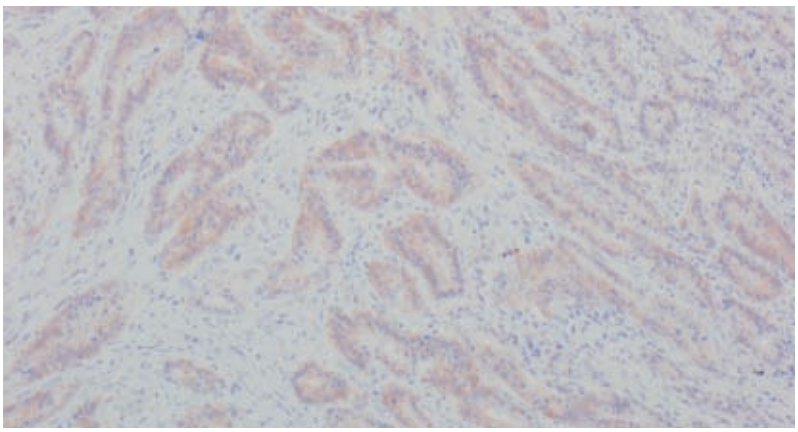


Figure 51
Gastric cancer. Score 2+
10x magnification.

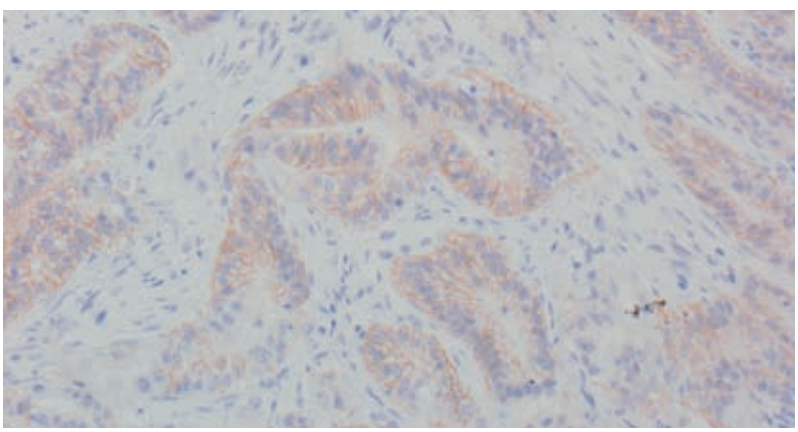


Figure 52
Gastric cancer. Score 2+
20x magnification.

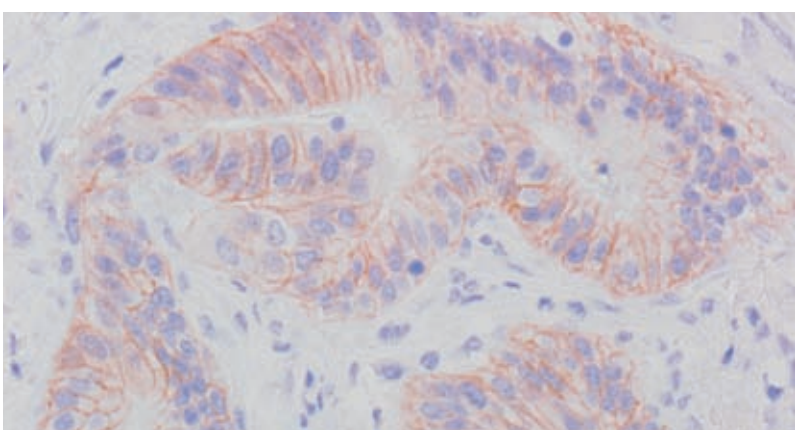


Figure 53
Gastric cancer. Score 2+
40x magnification.

HercepTest™ Score 2+

The tumor cells exhibit complete, basolateral or lateral membrane staining; the intensity is weak to moderate.

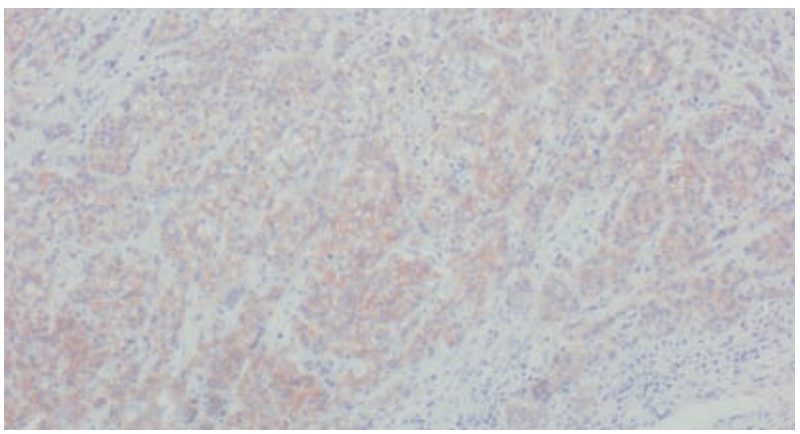


Figure 54
Gastric cancer. Score 2+
10x magnification.

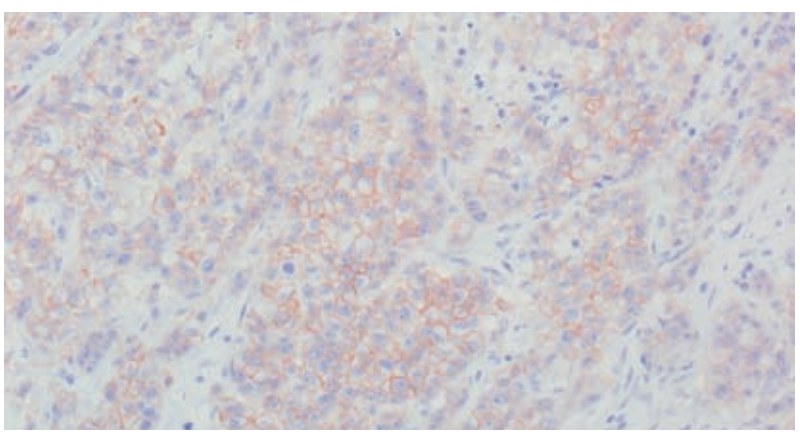


Figure 55
Gastric cancer. Score 2+
20x magnification.

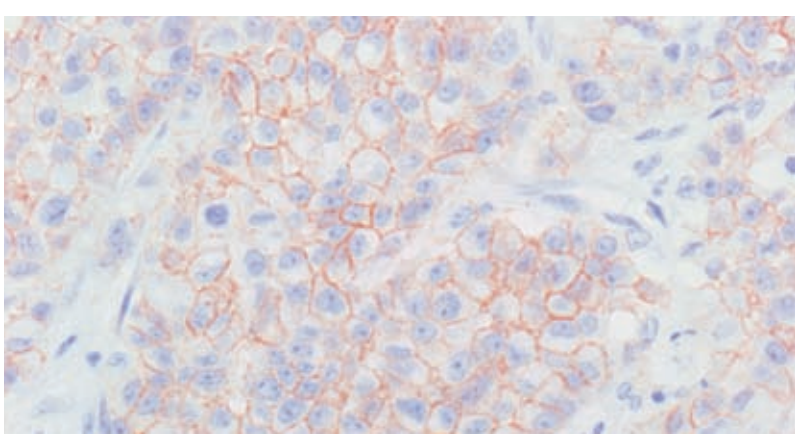


Figure 56
Gastric cancer. Score 2+
40x magnification.

HercepTest™ Score 2+

These infiltrating tumor cells exhibit complete, basolateral or lateral membrane staining; the intensity is weak to moderate.

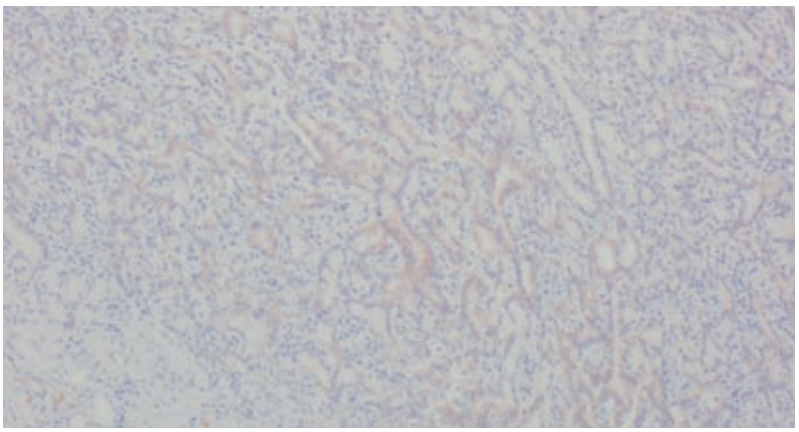


Figure 57
Gastric cancer. Score 2+
10x magnification.

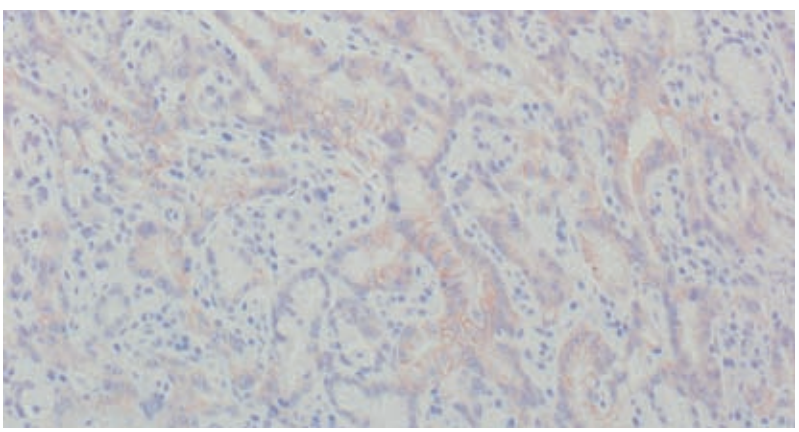


Figure 58
Gastric cancer. Score 2+
20x magnification.

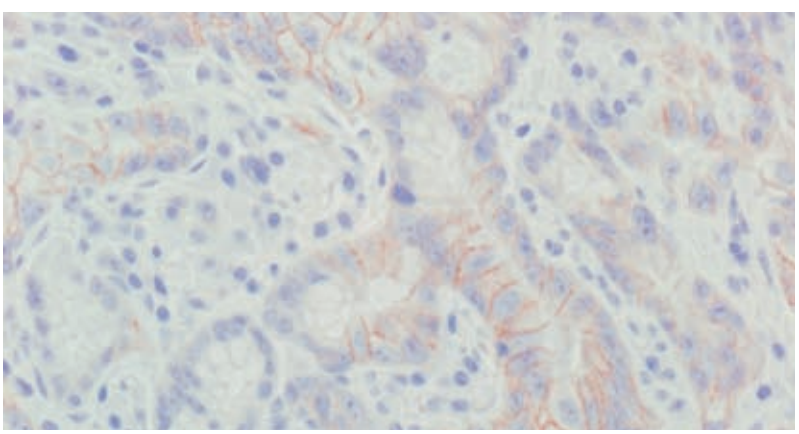


Figure 59
Gastric cancer. Score 2+
40x magnification.

HercepTest™ Score 2+

The tumor cells exhibit complete, basolateral or lateral membrane staining; the intensity is weak to moderate.

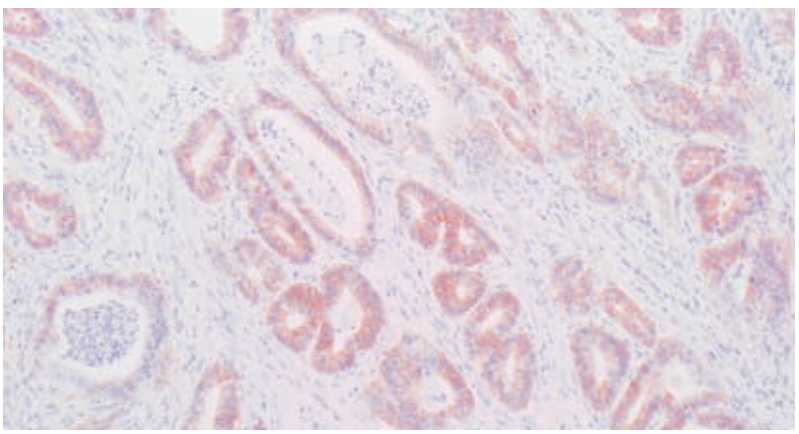


Figure 60
Gastric cancer. Score 2+
10x magnification.

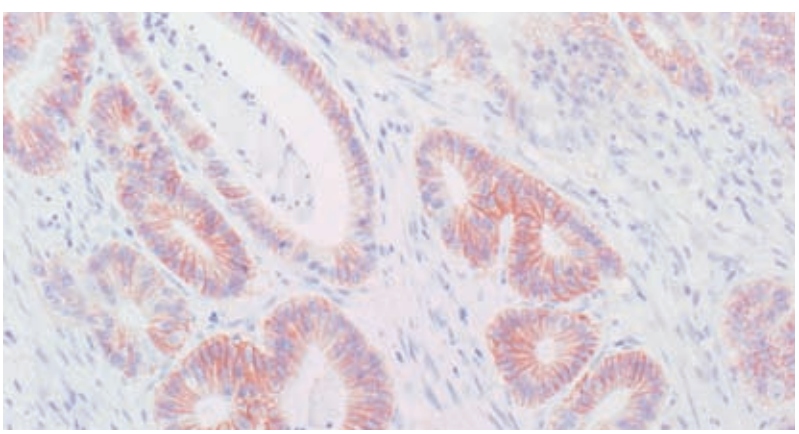


Figure 61
Gastric cancer. Score 2+
20x magnification.

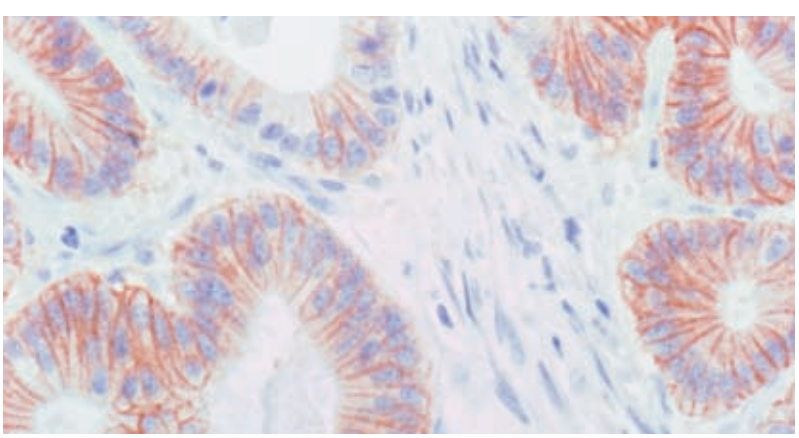


Figure 62
Gastric cancer. Score 2+
40x magnification.

HercepTest™ Score 3+

The tumor cells exhibit strong, complete, basolateral or lateral membrane staining.

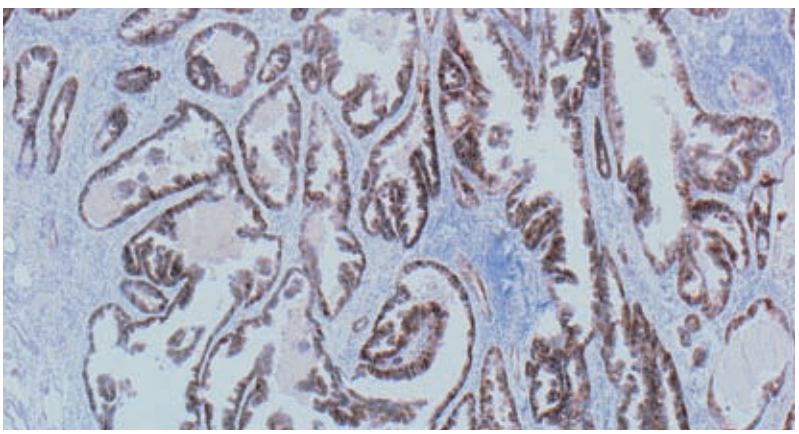


Figure 63
Gastric cancer. Score 3+
4x magnification.

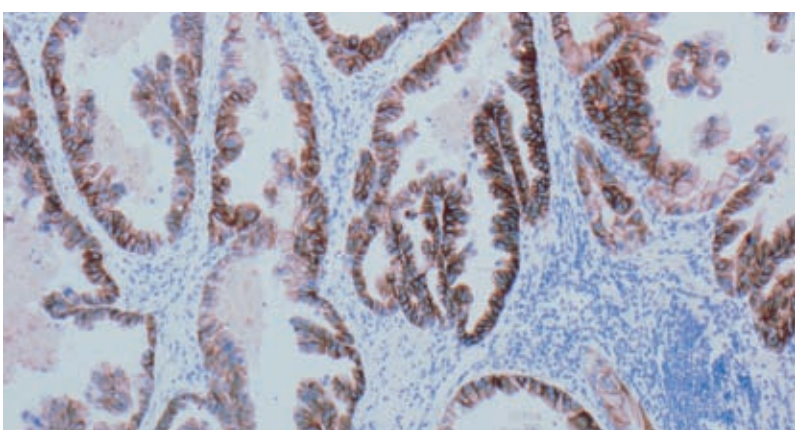


Figure 64
Gastric cancer. Score 3+
10x magnification.

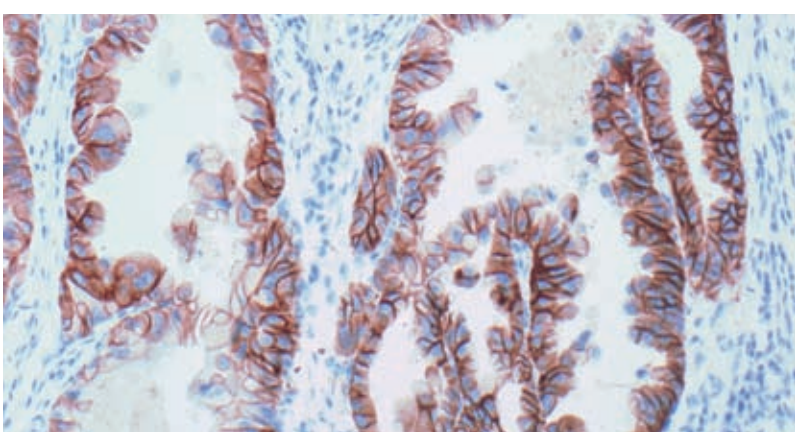


Figure 65
Gastric cancer. Score 3+
20x magnification.

HercepTest™ Score 3+

The tumor cells exhibit strong, complete, basolateral or lateral membrane staining.

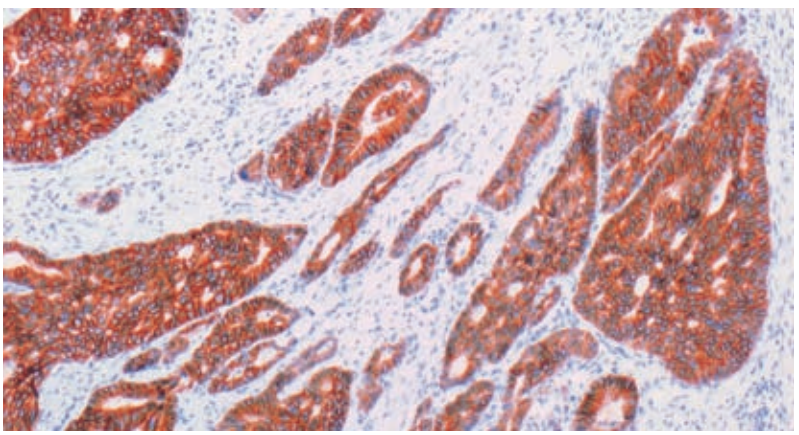


Figure 66
Gastric cancer. Score 3+
10x magnification.

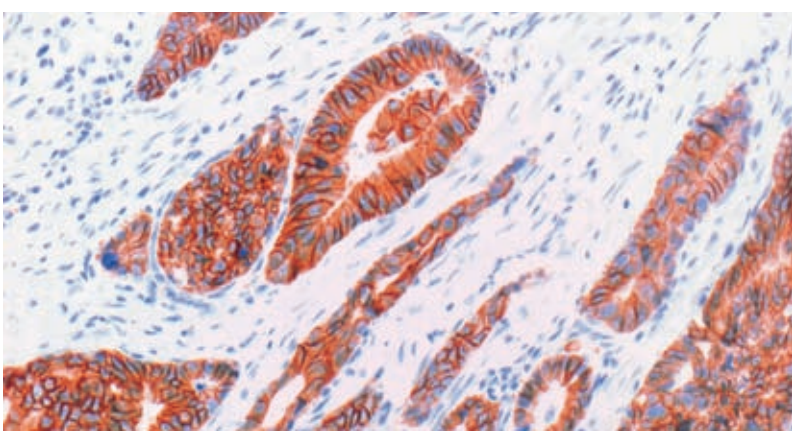


Figure 67
Gastric cancer. Score 3+
20x magnification.

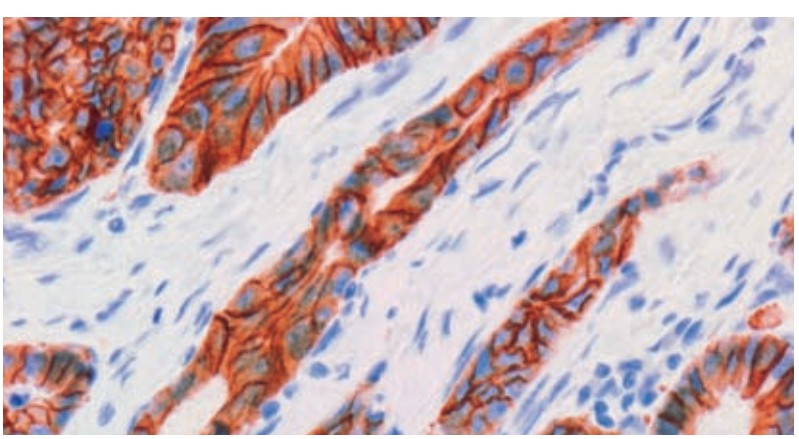


Figure 68
Gastric cancer. Score 3+
40x magnification.

HercepTest™ Score 3+

The tumor cells exhibit strong, complete, basolateral or lateral membrane staining.

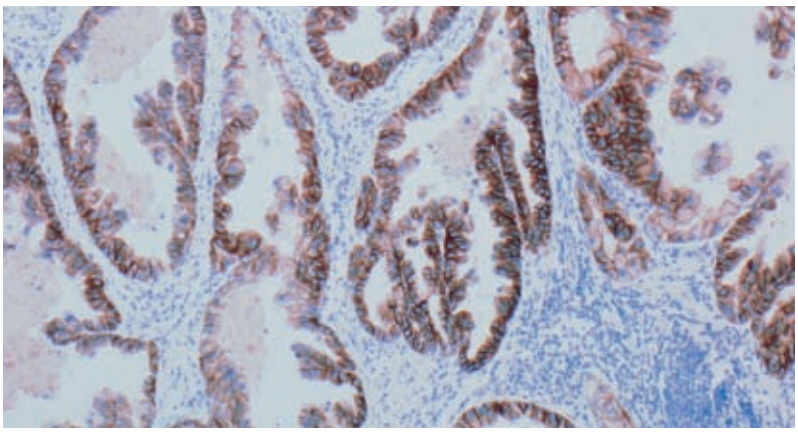


Figure 69
Gastric cancer. Score 3+
10x magnification.

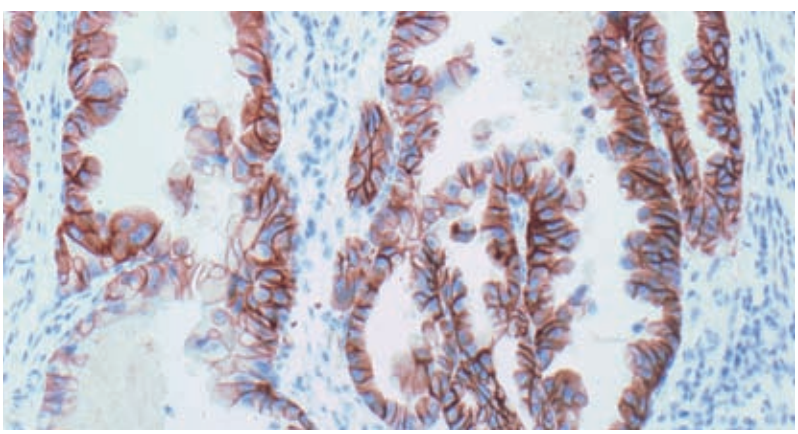


Figure 70
Gastric cancer. Score 3+
20x magnification.

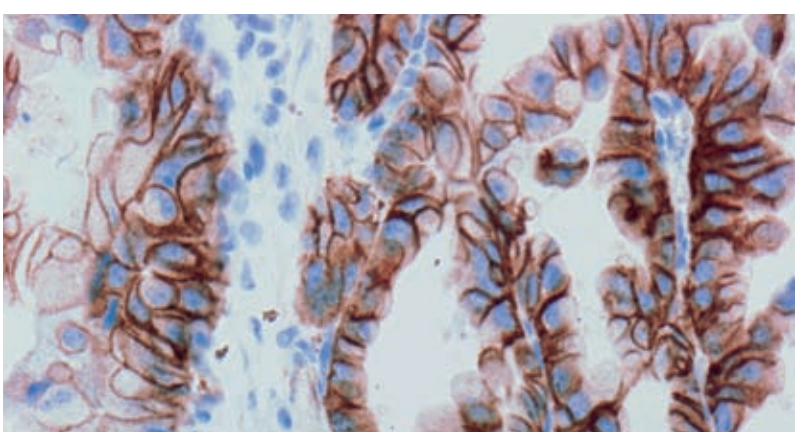


Figure 71
Gastric cancer. Score 3+
40x magnification.

HercepTest™ Score 3+

Tumor cells exhibit strong, complete, basolateral or lateral membrane staining.

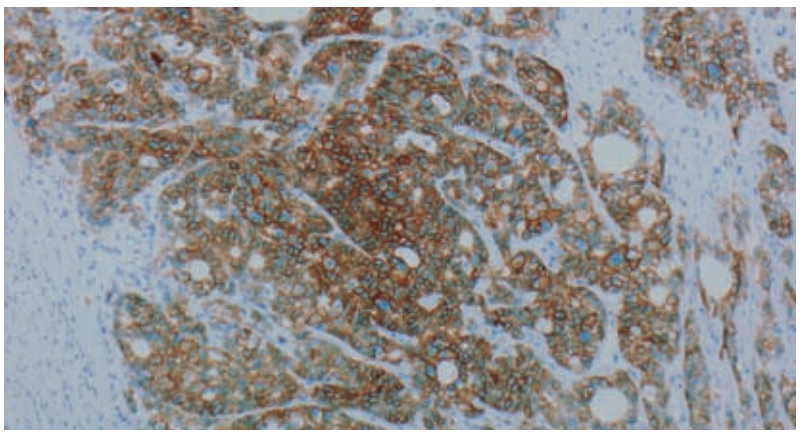


Figure 72
Gastric cancer. Score 3+
10x magnification.

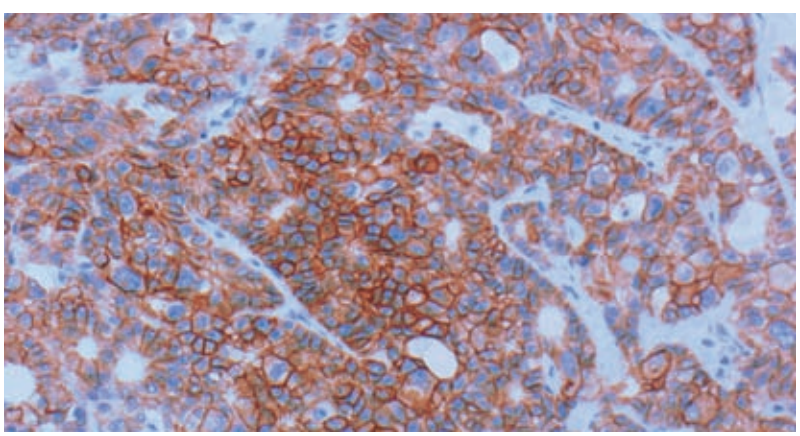


Figure 73
Gastric cancer. Score 3+
20x magnification.

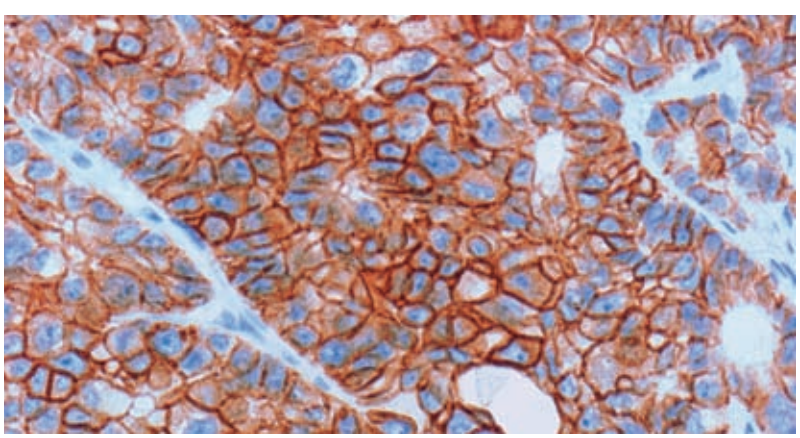


Figure 74
Gastric cancer. Score 3+
40x magnification.

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Corporate Headquarters
Denmark
+45 44 85 95 00

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+31 20 42 11 100

Norway
+47 23 14 05 40

Poland
+48 58 661 1879

Spain
+34 93 499 05 06

Sweden
+46 8 556 20 600

Switzerland
+41 41 760 11 66

United Kingdom
+44 (0)1 353 66 99 11

United States of America
+1 805 566 6655

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