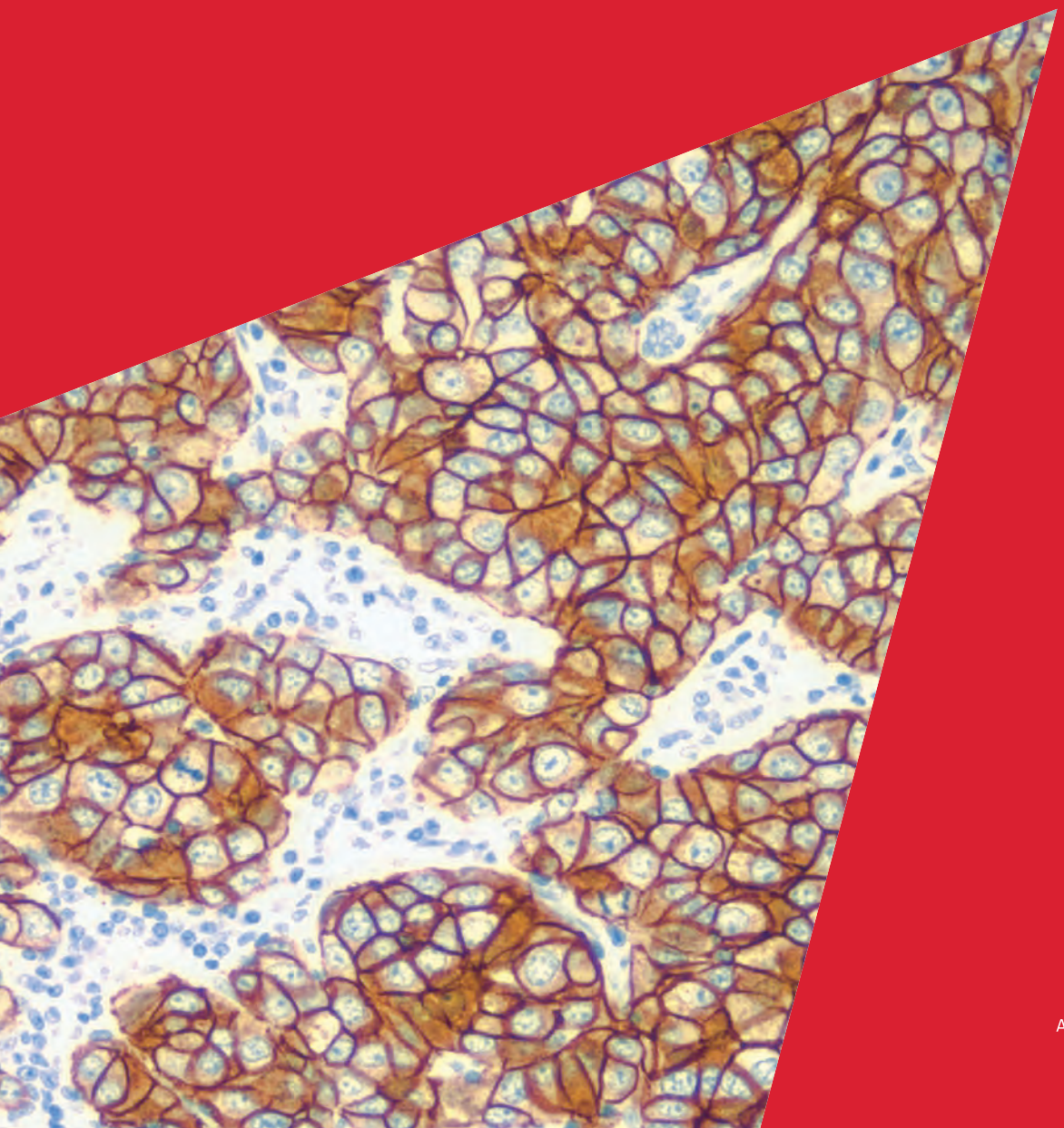
EDUCATION

HercepTest™ | Interpretation Manual Breast Cancer

Dako
A Better Path™



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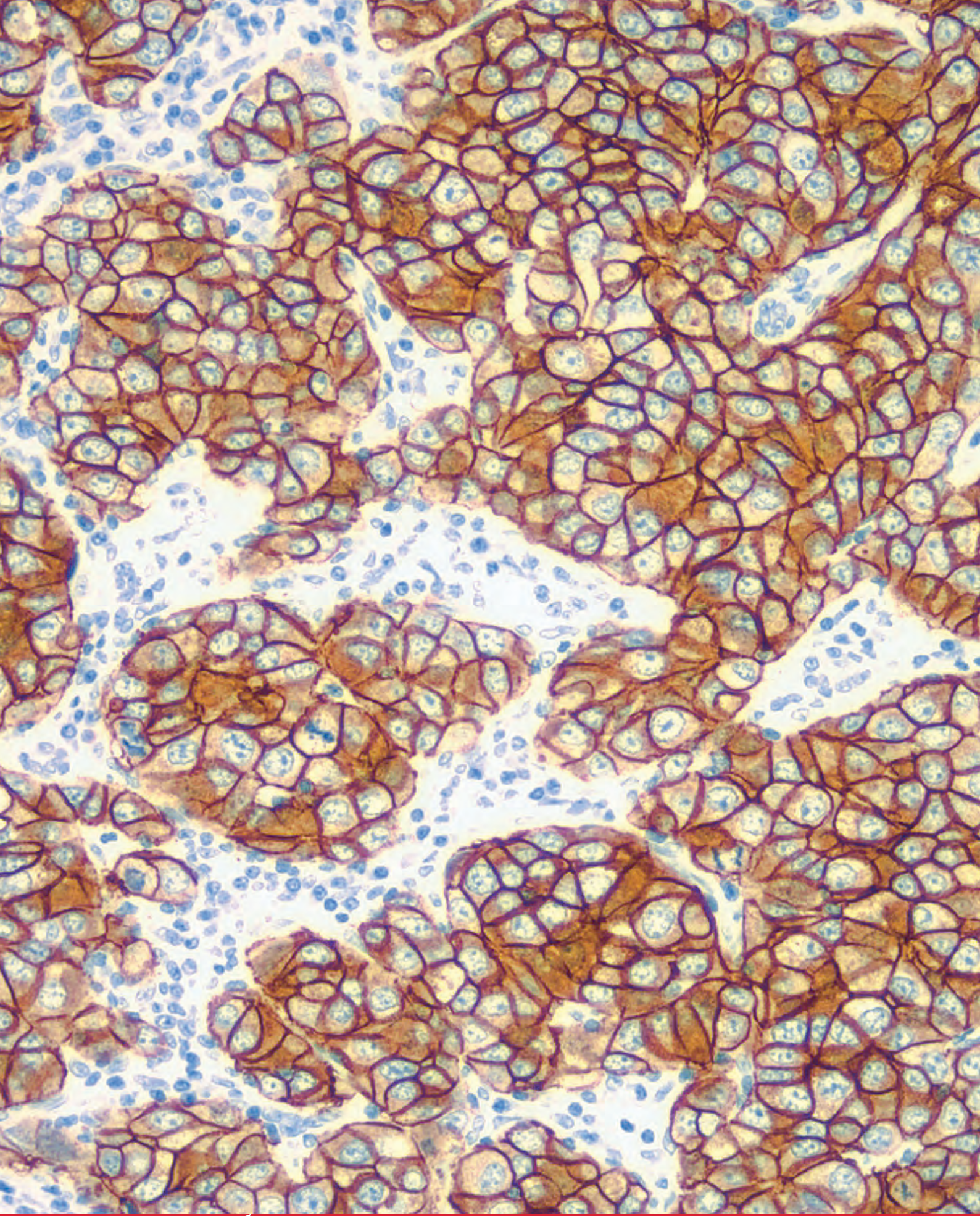


Introduction

HercepTest™ Interpretation Manual

HercepTest™ is a semi-quantitative immunohistochemical assay to determine HER2 protein overexpression in breast cancer tissues routinely processed for histological evaluation and formalin-fixed, paraffin-embedded cancer tissue from patients with adenocarcinoma of the stomach, including the gastroesophageal junction*. HercepTest™ is indicated as an aid in the assessment of breast and gastric cancer patients for whom Herceptin® (trastuzumab) treatment is being considered (see Herceptin® package insert).

HercepTest™ Interpretation Guidelines

Prior to HercepTest™, immunohistochemistry was practiced largely as a subjective method, ideally suited for qualitative analysis. HercepTest™, however, changed this paradigm, as the determination of positivity was no longer a simple yes or no answer. Patients are now evaluated using immunohistochemistry technology applied as a semi-quantitative tool with a scoring system reflective of intensity of staining in conjunction with percentage of stained tumor cells. This shift in application introduced a change in the way immunohistochemistry was viewed.

This HercepTest™ Interpretation Manual for breast cancer 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 breast carcinomas stained with HercepTest™. Example cases of various staining intensities of HER2 expression 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 will provide a solid foundation for evaluating slides stained with HercepTest™.

Most metastatic breast cancer tissue specimens tested for HER2 overexpression are scored with either 0 or 3+ staining intensity. While the majority of cases are clear-cut, a small percentage of the remaining 1+ and 2+ scored samples may be more difficult to interpret. In this manual, we will focus on these equivocal samples. In addition, we will review images of sample artifacts and discuss how to best interpret such cases.

***HER2* IQFISH pharmDx™**

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

Photomicrographs

The included photomicrographs are breast carcinoma unless otherwise noted.

* *Van Cutsem E, Kang Y, Chung H, Shen L, Sawaki A, Lordick F, et al. Efficacy results from the ToGA trial: A phase III study of trastuzumab added to standard chemotherapy in first-line human epidermal growth factor receptor 2 positive advanced gastric cancer. J Clin Oncol 2009;27:18s, (suppl; abstr LBA2409). (http://media.asco.org/silver).*

HER2 Overview

HER2 Protein and HER2 Family

The gene encoding HER2 is located on chromosome 17 and is a member of the EGF/erbB growth factor receptor gene 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 generally occurs when the ligand and a dimer of the same monomer or other member of the HER family are bound together. HER2 has no known ligand. 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-overexpression cells directly contribute to the pathogenesis and clinical aggressiveness of tumors.* This overexpression is associated with poor prognosis, including reduced relapse-free and overall survival.

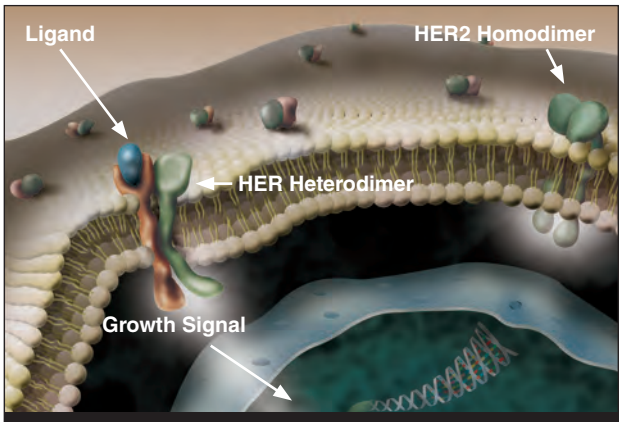
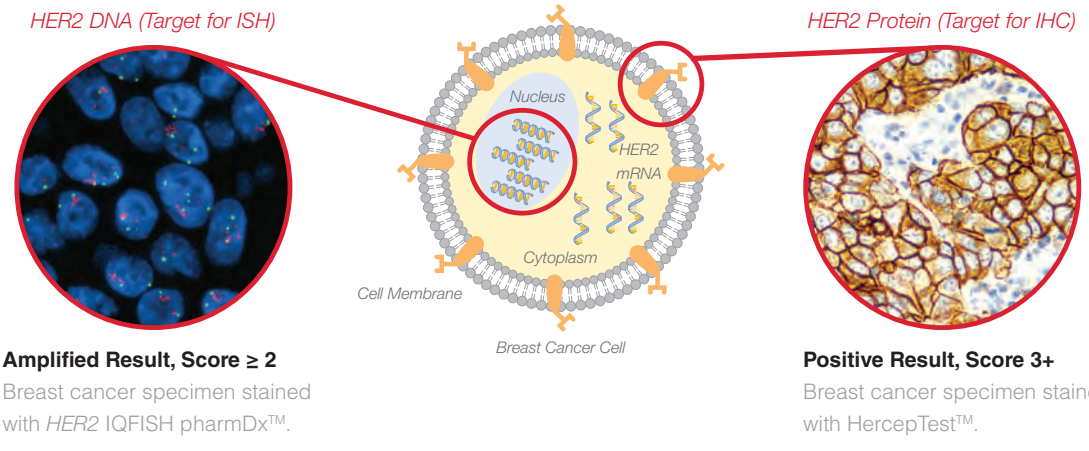


Figure 1: Representation of HER family

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
Breast cancer specimen stained with *HER2* IQFISH pharmDx™.

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

Figure 2: IHC and ISH targets for HER2 testing

* *Robert W. Carlson, MD; Susan J. Moench, et al. HER2 Testing in Breast Cancer: NCCN Task Force Report and Recommendations: Journal of the National Comprehensive Cancer Network July 2005. | Edith A. Perez, Vera J. Suman, et al. HER2 Testing by Local, Central and Reference Laboratories in Specimens from the North Central Cancer Treatment Group N9831 Intergroup Adjuvant Trial. Journal of Clinical Oncology July 1, 2006. | Martine J. Piccart-Gebhart, MD., Ph.D., Marion Proctor, M. Sci., et al. Trastuzumab after Adjuvant Chemotherapy in HER2-Positive Breast Cancer. New England Journal of Medicine October 20, 2005.*

HER2 Testing Algorithm

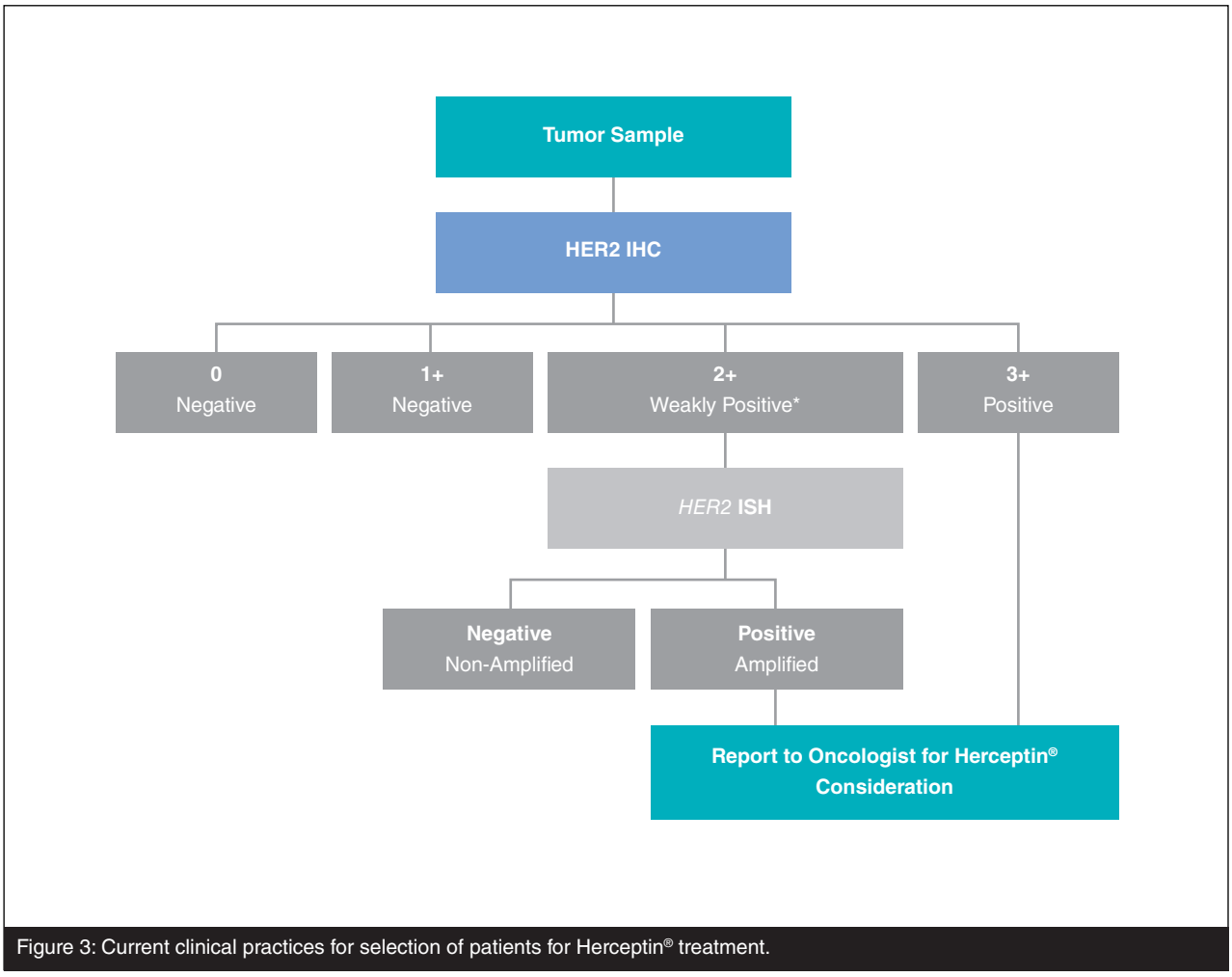


Figure 3: Current clinical practices for selection of patients for Herceptin® treatment.

* *For Herceptin® – Weakly positive cases (2+) may be considered equivocal and reflexed to ISH testing.*

NCCN Practice Guidelines in Oncology, CAP Conference Summary Laboratories performing HER2 testing should meet quality assurance standards.

The HercepTest™ Kit

HercepTest™ 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 gastro-esophageal 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.

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)

- Mayer's Hematoxylin for Dako Autostainer/ Autostainer Plus, Code S3301
- Mayer's Hematoxylin for Automated Link Platforms, Code SK308

Three HercepTest™ kit configurations are available:

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

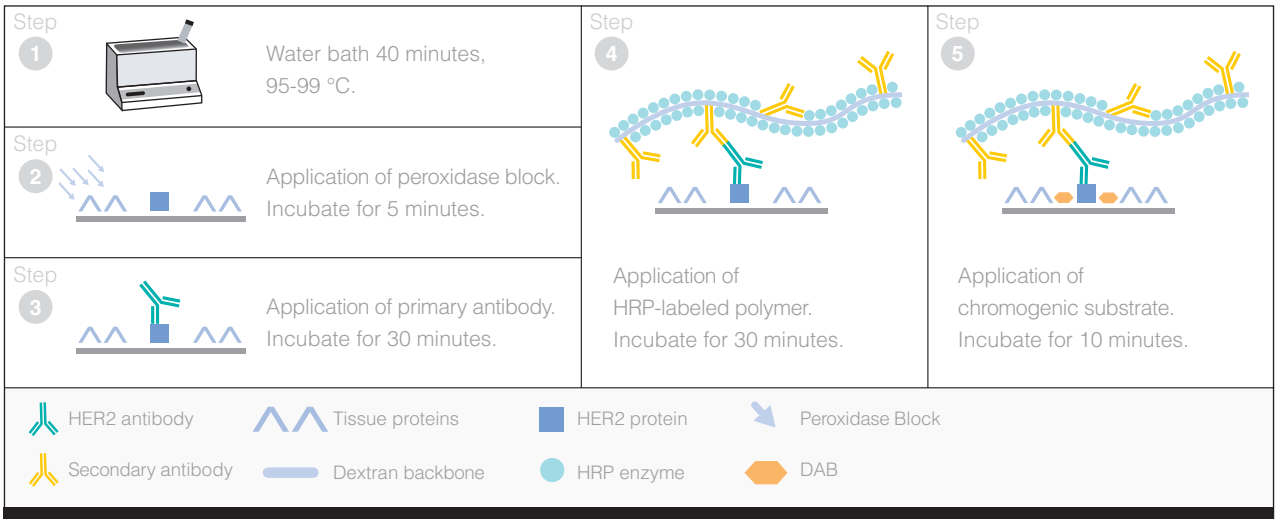


Figure 4: HercepTest™ procedure

HER2 IQFISH pharmDx™ Kit

HER2 IQFISH pharmDx™ kit is a direct fluorescence in situ hybridization (FISH) assay designed to quantitatively determine *HER2* gene amplification in formalin-fixed, paraffin-embedded (FFPE) breast cancer tissue specimens and FFPE specimens from patients with adenocarcinoma of the stomach, including gastroesophageal junction. *HER2* IQFISH pharmDx™ kit is indicated as an aid in the assessment of breast and gastric patients for whom Herceptin® (trastuzumab) treatment is being considered (see Herceptin® package insert).

For breast cancer patient, results from the *HER2* IQFISH pharmDx™ Kit are intended for use as an adjunct to the clinicopathologic information currently used for estimating prognosis in stage II, node-positive breast cancer patients.

The assays includes a chromosone 17 reference probe to correct for *HER2* signal number in the event of chromosone 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)

HER2 IQFISH pharmDx™ is a complete kit and includes

- Pre-Treatment Solution 20x
- Pepsin, Ready-to-Use
- Pepsin Diluent (10x)
- *HER2*/CEN-17 IQISH Probe Mix
- Stringent Wash Buffer 20x
- Fluorescence Mounting Medium, containing DAPI
- Wash Buffer 20x
- Coverslip Sealant

K5731		**20 Tests**
HER2 IQFISH pharmDx™ Kit (22 x 22 mm target area)		

Hybridizer Instrument for In Situ Hybridization (FISH)

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

S2450	Hybridizer	**120 volt**
S2451	Hybridizer	**240 volt**

Figure 5: Dako Hybridizer instrument

Checklist

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 or Bouin's fixative only?	☐	☐
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 for five minutes and specimen fully covered?	☐	☐
Specimens fully covered for 30 minutes with three drops (100 µl) of Primary Antibody or Negative Control Reagent?	☐	☐
Visualization Reagent applied for 30 minutes and specimen fully covered?	☐	☐
Substrate-Chromogen (DAB) Solution prepared properly? 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 programming 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. Dako Automated Link Platform: Mix an appropriate 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:

_____

_____

_____

Recommendations

Recommended Data Tracking for HercepTest™ Immunostaining

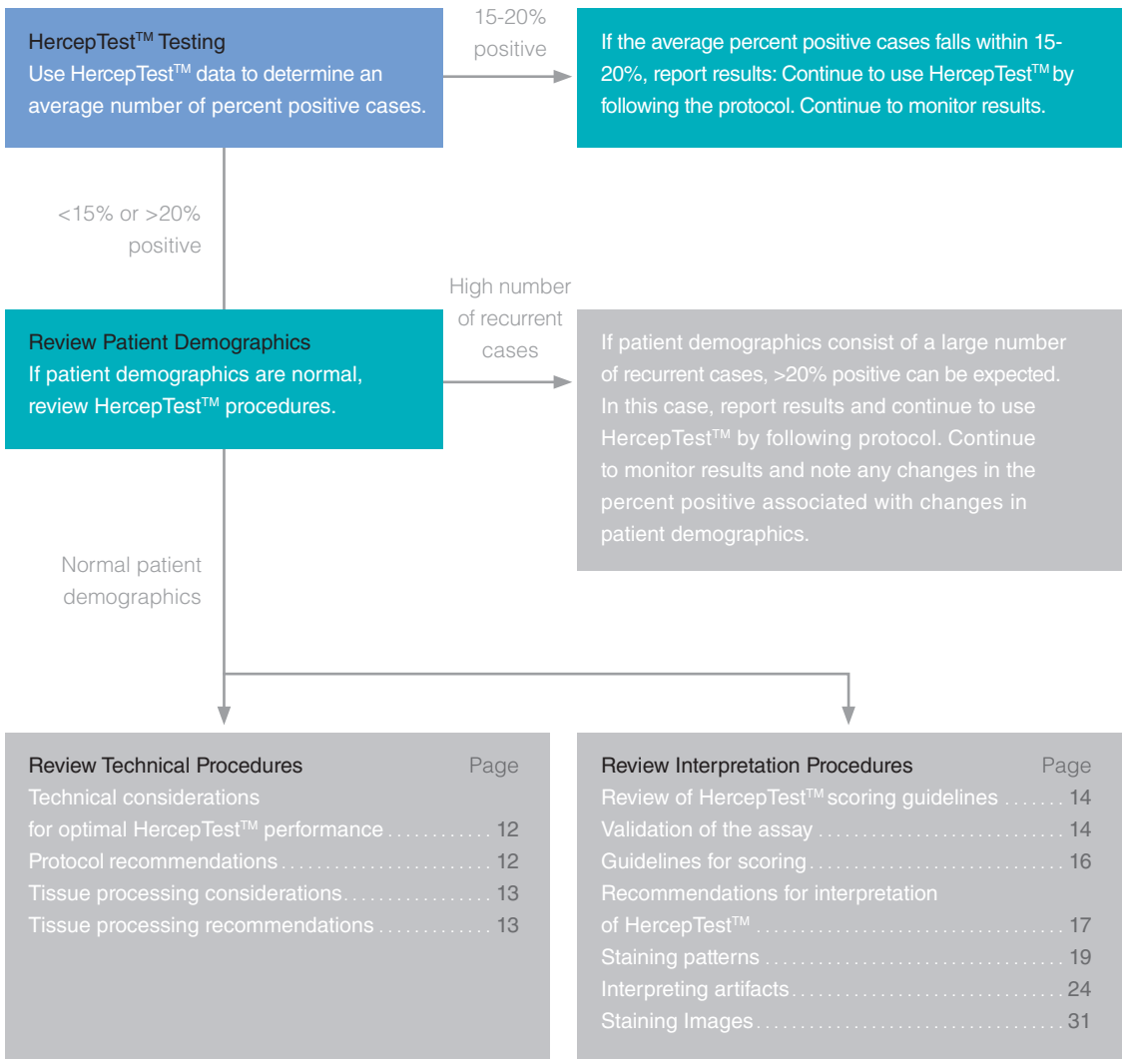


Table 1

Technical Considerations

Technical Considerations for Optimal HercepTest™ Performance

While accurate and consistent interpretation can be achieved, technical issues relating to the performance of HercepTest™ are not always easy to identify. If cumulative laboratory test results fall outside the expected range of 15-20% positive, evaluate the patient demographics and then address any technical problems.

Technical problems 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 PT Link

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. Dedicated PT Link equipment must be used for HercepTest™.

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 an Autostainer Link or a Dako Autostainer. 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
- Bouin's Solution

Fixation Times

Neutral Buffered Formalin:

- 18-24 hours

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

Bouin's:

- 1-12 hours depending on tissue thickness

Tissues fixed in Bouin's solution must be washed in 70% ethanol to remove picrates prior to aqueous washes. Bouin's solution may not be optimal, if FISH testing is needed.

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. Properly fixed and embedded tissues expressing the HER2 protein will keep indefinitely prior to sectioning and slide mounting if stored in a cool place, 15-25 °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 specimens should be cut into sections of 4-5 µm thickness.

To achieve reproducible results, each laboratory performing HercepTest™ should monitor its rate of positivity. If the positive rate exceeds 20%, a complete review of interpretation and technical procedures should be done.

Guidelines

Review of HercepTest™ Scoring Guidelines for Breast Tissue

HercepTest™ is a semi-quantitative immunohistochemical assay to determine HER2 protein overexpression in breast cancer tissues routinely processed for histological evaluation.

For the determination of HER2 protein overexpression, only the membrane staining intensity and pattern should be evaluated using the scale presented on page 16. Slide evaluation should be performed using a light 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 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 tissues and proper staining technique. The ideal positive tissue control is weakly positive staining tissue. 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.

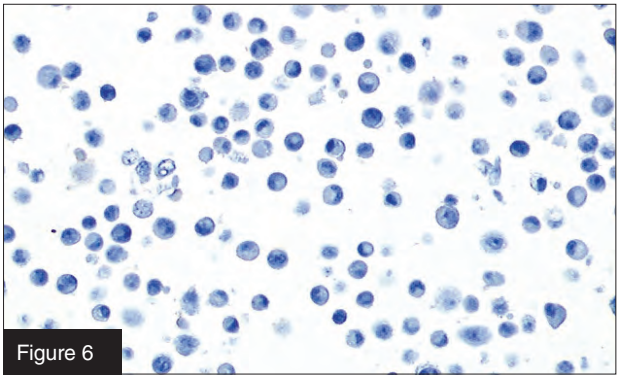


Figure 6

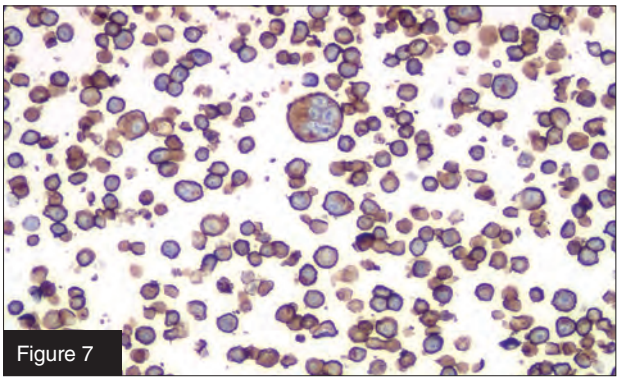


Figure 7

Figure 6: 0 control cell line, MDA-231, stained with HercepTest™. No staining of the membrane is observed. *(20x magnification).*

Figure7: 3+ control cell line, SK-BR-3, stained with HercepTest™. A strong staining of the entire membrane is observed. *(20x magnification).*

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 (Fig. 8, 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 (Fig. 8, 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 (Fig. 8, 2). In addition, in some cells dot-like immunostaining can be observed in the Golgi region of the cytoplasm (Fig. 8, 3).

The different categories of HER2-specific cellular stainings may be reflected in the different appearances of acceptable 1+ cellular staining runs, e.g. low (Fig. 9) and moderate (Fig. 10).

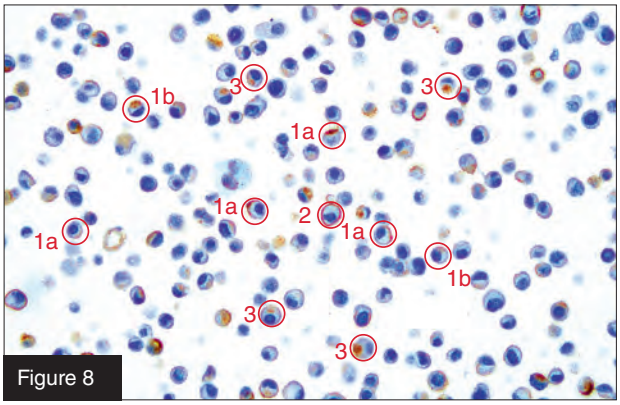


Figure 8

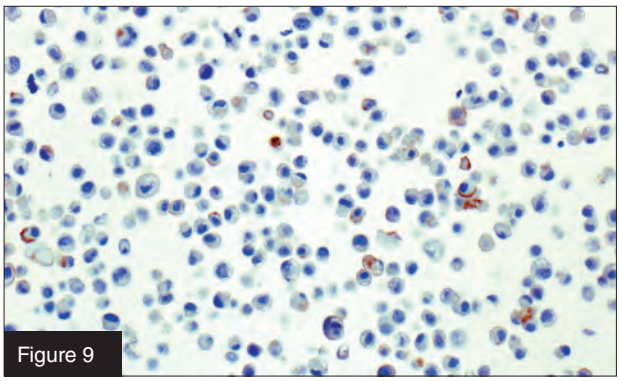


Figure 9

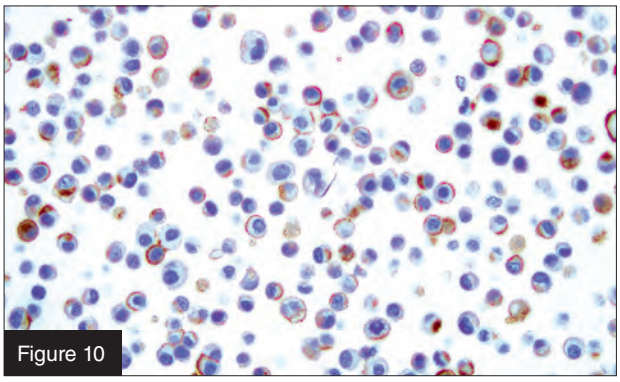


Figure 10

Figure 8: 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 9: 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 10: 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 always be performed within the context of the pathologist's past experience and best judgment in interpreting IHC stains. Only patients with invasive breast carcinoma should be scored. In cases with carcinoma in situ and invasive carcinoma in the same specimen, only the invasive component should be scored. Figure 9 shows examples of staining patterns.

Score to Report	HER2 Protein Overexpression Assessment	Staining Pattern
0	Negative	No staining is observed, or membrane staining is observed in <10% of the tumor cells.
1+	Negative	A faint/barely perceptible membrane staining is detected in >10% of tumor cells. The cells exhibit incomplete membrane staining.
2+	Weakly Positive*	A weak to moderate complete membrane staining is observed in >10% of tumor cells.
3+	Positive	A strong complete membrane staining is observed in >10% of tumor cells.

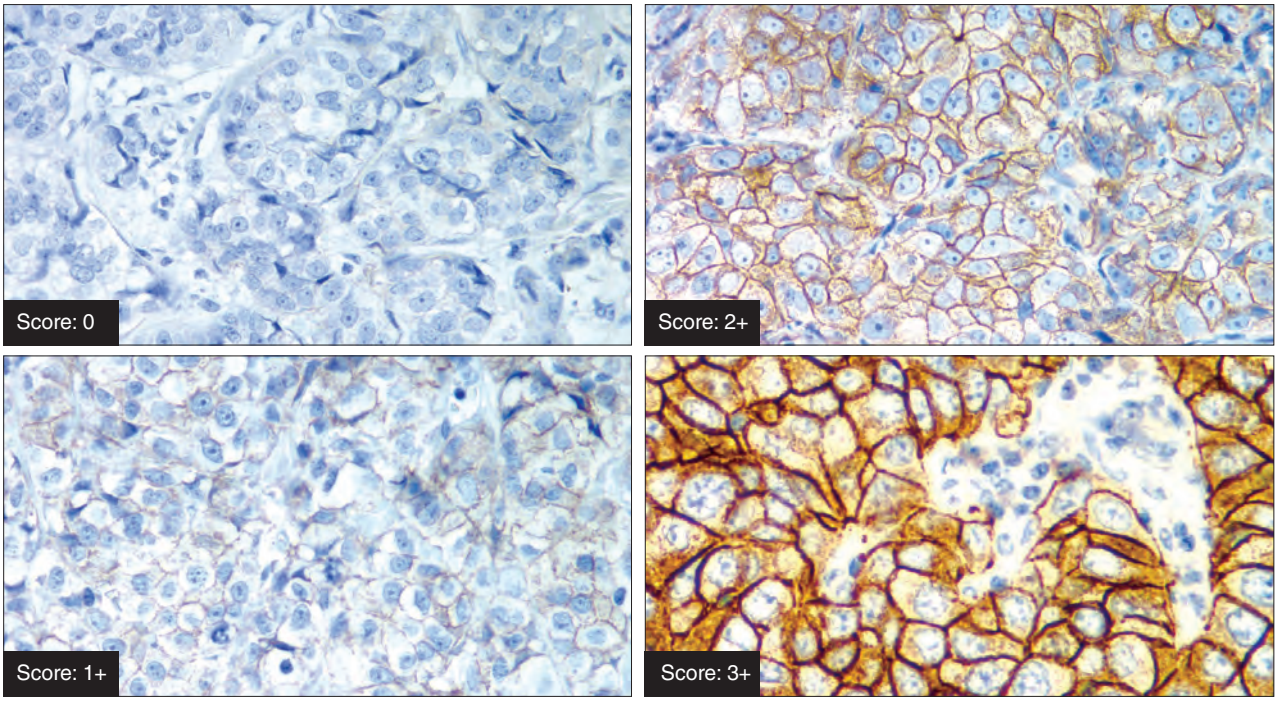


Figure 11: Examples of staining patterns for tissue scored 0, 1+, 2+, and 3+, at *(40x magnification)*.

* *Weakly positive cases (2+): may be considered equivocal and reflexed to ISH testing.*

Interpretation

Recommendations for Interpretation of HercepTest™ – Breast Cancer

Dako emphasizes that scoring of HercepTest™ must be performed in accordance with the guidelines established 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.

The original Immunohistochemical assay (CTA) used by Genentech for the Herceptin® clinical trials utilized a scoring system later adopted by Dako as an integrated part of HercepTest™.

Steps for HercepTest™ Interpretation

Manual or Automated Interpretation

1 Evaluate the control cell lines to validate the assay run.

2 Next, evaluate the positive and negative control slides.

3 An H&E stained section of the tissue sample is recommended for the first evaluation. (The tumor may not be obvious when looking at the sample stained with HercepTest™. An H&E stain allows the pathologist to verify the presence of the invasive tumor).

Manual Interpretation with Conventional Microscopy

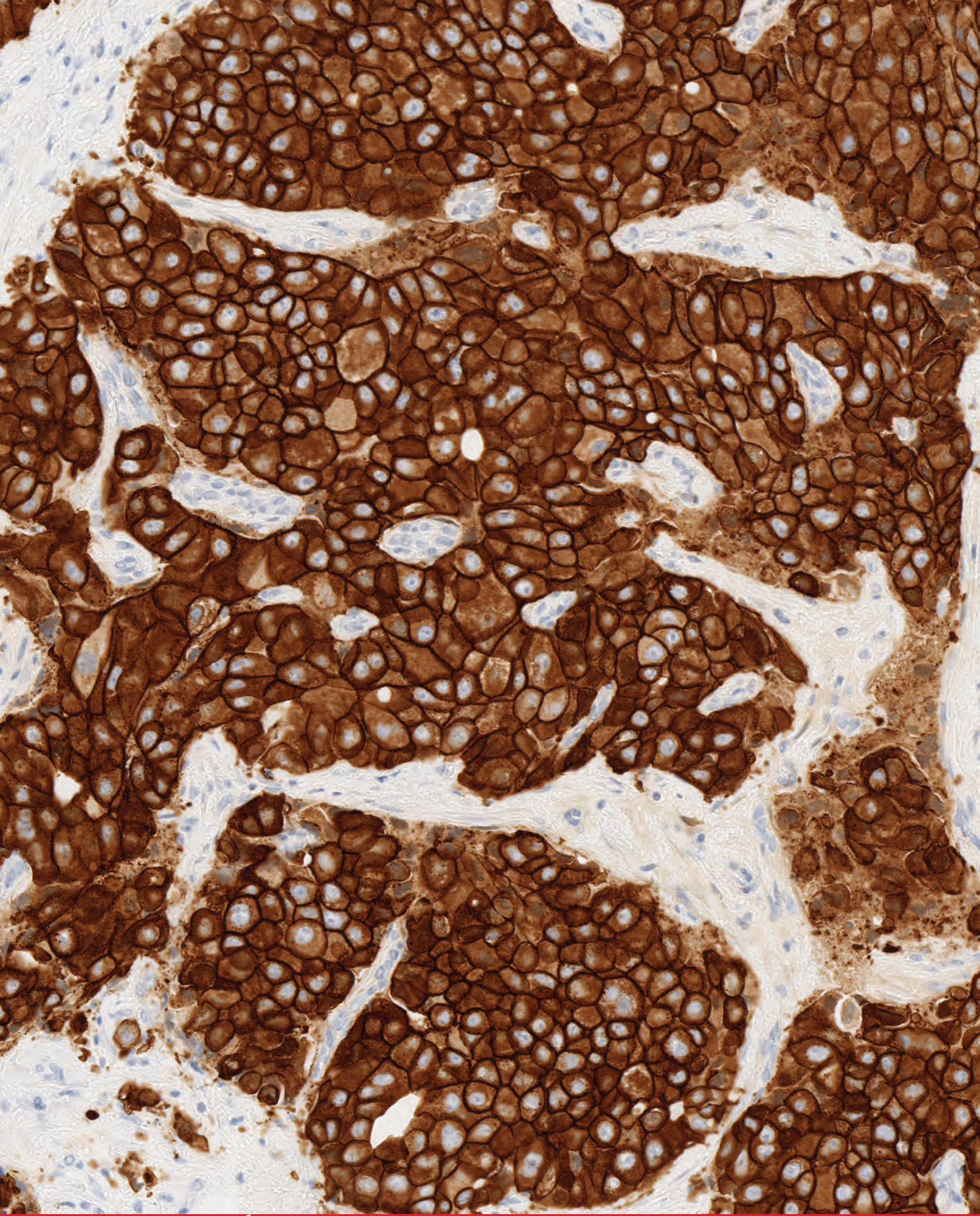
1 Evaluate the HER2 sections for estimation of the percentage of tumor cells showing membrane staining at low power first, 4x magnification. The majority of strongly positive cases will be obvious at 4x magnification. Invasive (infiltrating) breast cancer tumor cells are the only component that should be scored. In situ breast cancer cells should not be scored.

2 To verify the percentage of stained tumor cells and completeness of membrane staining, use 10x magnification. Well-preserved and well-stained areas of the specimen should be used to make a determination of the percent of positive infiltrating tumor cells.

3 If determination of equivocal 1+/2+ cases is difficult using 10x magnification, confirm score using 20x or 40x magnification.

4 If there is complete membrane staining at a weak to moderate intensity in greater than 10% of the tumor cells, the score of the specimens is 2+. This is usually accompanied by incomplete membrane staining of the majority of the remaining tumor cells.

5 In the majority of 3+ cases, staining is usually homogeneous with approximately 80% of the tumor cells positive with intense membrane staining.



Staining Patterns

Heterogeneous Staining

Heterogeneous staining patterns occur less frequently as true biological entities. Consequently, when present, this staining pattern may represent artifacts of tissue preparation.

- The pathologist's experience and judgment is important in the evaluation of heterogeneous staining.
- Review these cases at a low power on the microscope.
- If the staining pattern is an artifact, the best representative area(s) should be graded. There must be >10% of the infiltrative tumor cells demonstrating complete membrane staining for the score to be at least 2+ or greater.
- In the absence of clear evidence for biological heterogeneity, the best representative area(s) should be scored, as long as >10% of the infiltrative tumor cells in these areas demonstrate complete membrane staining at a moderate to strong intensity. Focus on the most well-preserved and well-stained areas to make the determination.

Focal Staining

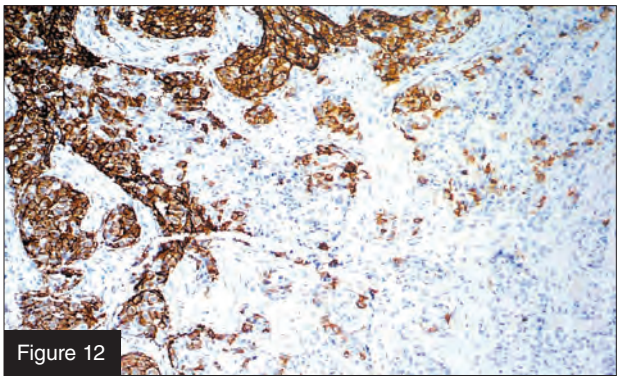
Focal staining is usually 1+. Focal staining usually occurs in <10% of tumor cells and the score is, therefore, no greater than 1+. By definition, focal staining implies that most of the tumor cells are not stained or are stained only partially on their membranes. However, it is important to verify that fewer than 10% of the tumor cells demonstrate complete membrane staining.

Staining not Associated with Tumor Cells

Occasionally, HER2 staining can be observed as luminal secretions of normal breast epithelium or may be seen as extracellular accumulations within the tissue. This staining pattern should be disregarded.

DCIS Cases

HercepTest™ has no indication for ductal carcinoma in situ (DCIS) at this time. Staining of DCIS should be disregarded.



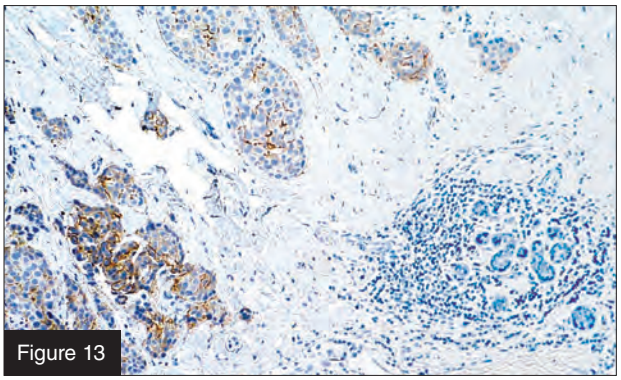


Figure 12: Breast carcinoma with example of heterogeneous staining. Characteristic feature: 3+ score on the left and 0 score on the lower right, with an intermingling of tumor cell subsets in between (>10% of the infiltrative tumor cells demonstrate complete membrane staining). *(4x magnification).*

Figure 13: Breast carcinoma with example of heterogeneous staining. Characteristic feature: 2+ score on lower left, 1+ score on upper middle, and negative on normal tissue on lower right. *(4x magnification).*

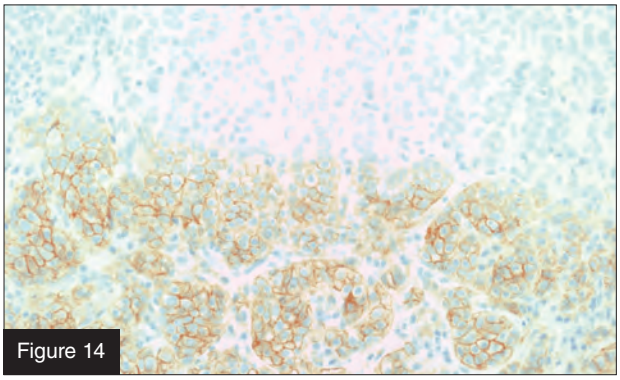


Figure 14

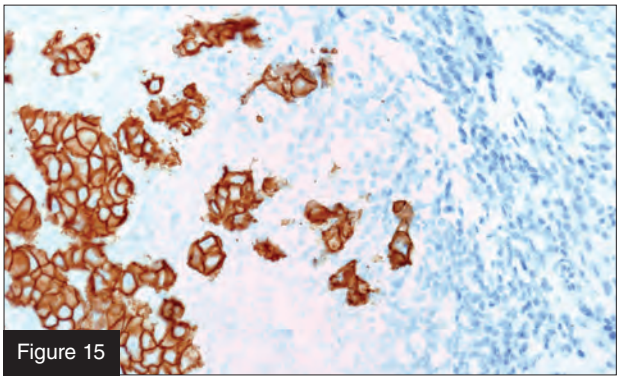


Figure 15

Figure 14: Breast carcinoma with example of heterogeneous staining due to incomplete spreading of reagent. Characteristic feature: 3+ score on the lower part and 0 score on the upper. *(10x magnification).*

Figure 15: Breast carcinoma with example of heterogeneous staining due to incomplete spreading of hematoxylin. Characteristic feature: Weak counterstain to the left, appropriate counterstain to the right. (*10x magnification).*

Artificial Heterogeneous Staining

Heterogeneous staining may occur as a consequence of suboptimal performance of the immunohistochemical test.

- Incomplete spreading of reagent

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 or Bouin's solution may be a source of background staining. Background staining with HercepTest™ is rare. This artifact may occur in 2-3% of cases. Background has been reported in breast tissues with abundant hyalinized stroma.

Possible Cause of Background

- Improper drying of slides (use a humid chamber for primary antibody/negative control and labeled polymer HRP reagent incubations when the assay is performed manually)
- Improper deparaffinization procedure
- Use of a different wash buffer than recommended (Code S3006 is recommended)
- Incomplete rinsing of reagents from slides

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

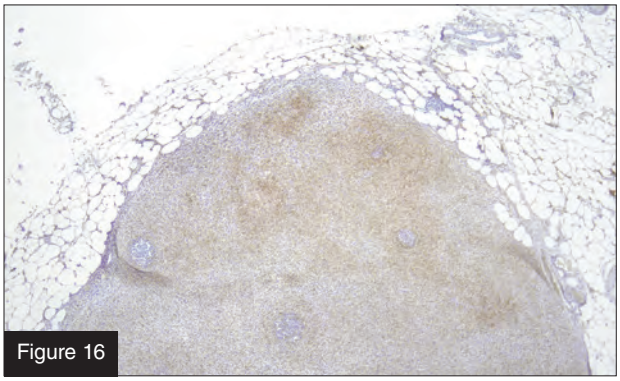


Figure 16

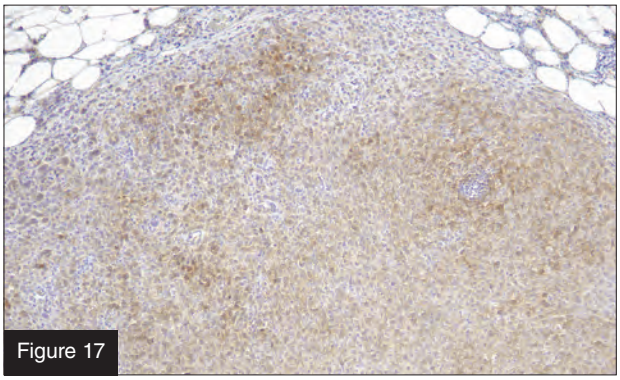


Figure 17

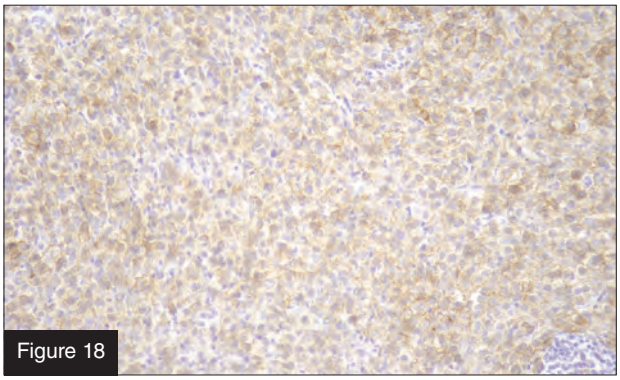


Figure 18

Example of high, non-specific background. Score: 0
Characteristic feature: Diffuse smudgy brown stain in background stroma and cells.

Figure 16: Breast carcinoma, brown staining is apparent. *(4x magnification).*

Figure 17: *Breast carcinoma, diffuse non-specific background staining can be seen. (10x magnification).*

Figure 18: *Breast carcinoma, minimal membrane staining is seen. 0 score is apparent. (20x magnification).*

Homogeneous Staining

Properly fixed breast cancer tissue with HER2 protein overexpression should reveal relative uniformity of immunostaining in individual tumor cells. In cases where variability in fixation of the tissue is present, the tissue may not appear homogeneous.

- In the majority of cases, breast tumor specimens stain homogenously for HER2.
- Evaluation of homogeneous staining should be based on an overall (average) of all the infiltrative tumor cells. Review average staining of the whole section.
- Carefully evaluate:
  - The percent of infiltrative tumor cells showing complete membrane staining.
  - The intensity of staining:
    - If >10% of the infiltrative tumor cells exhibit complete membrane staining and there is a moderate intensity of staining, the score would be at least 2+.
    - If it is difficult to determine whether >10% of the infiltrative tumor cells show complete membrane staining, the score should be no greater than 1+.

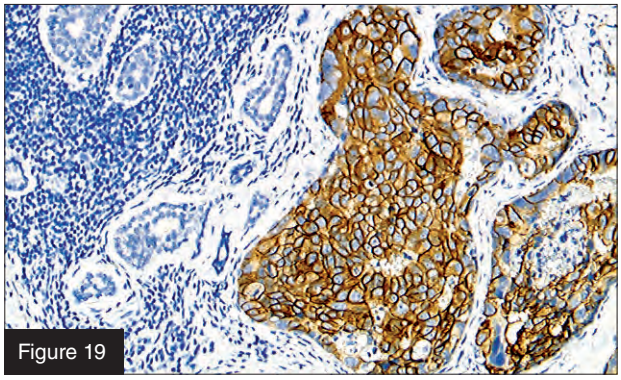
Staining of Normal Epithelium

Overexpression of HER2 on tumor cells is relative to a baseline level of expression on normal breast epithelium.

- Normal breast tissue rarely overexpresses HER2. Staining of normal ducts may be observed occasionally.
- The sensitivity of HercepTest™ has been established under controlled conditions to stain normal breast epithelium between 0-1+.
- If normal epithelium is staining >1+, the test should be repeated and the protocol should be observed closely.

This phenomenon may be caused by:

1. Fixatives other than Neutral Buffered Formalin or Bouin's solution.
2. Use of a steamer or microwave rather than a water bath for epitope retrieval.



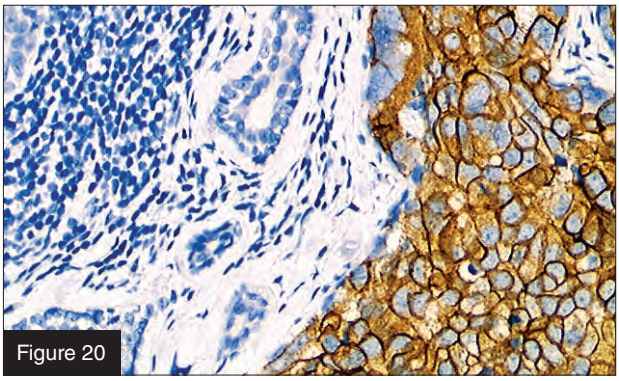


Figure 19: Breast carcinoma with no staining of normal ducts on the left and 3+ homogeneous staining on the right. *(10x magnification).*

Figure 20: Breast carcinoma with no staining of normal ducts on the left and 3+ homogeneous staining on the right. *(20x magnification).*

Cytoplasmic Staining – Homogeneous
Diffuse homogeneous staining is specifically confined to the cytoplasm. Score 0

Cytoplasmic Staining – "Dot" Artifact
The dot artifact is specific to the cytoplasm. This artifact is associated with tumors having neuroendocrine differentiation. Score 0

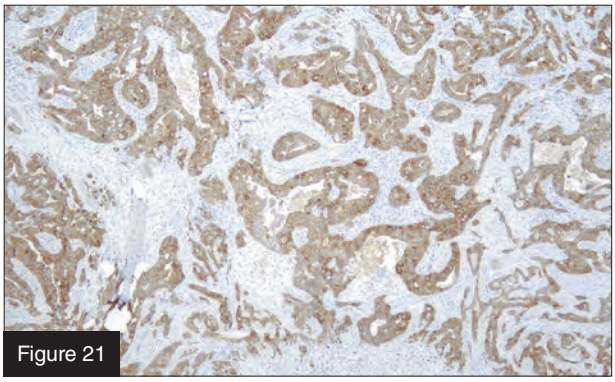


Figure 21

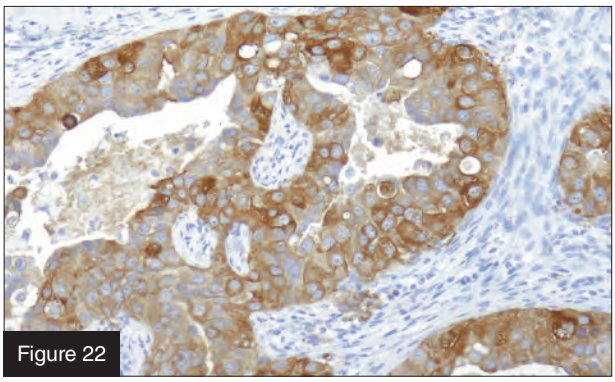


Figure 22

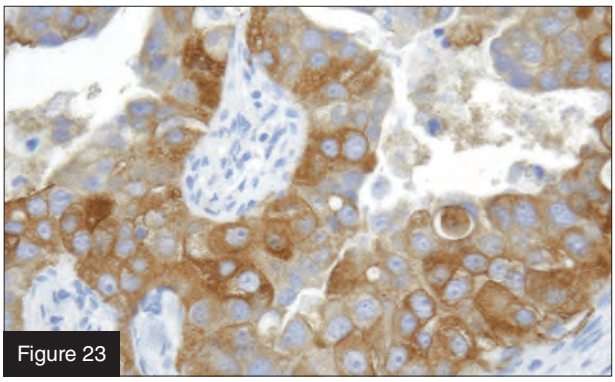


Figure 23

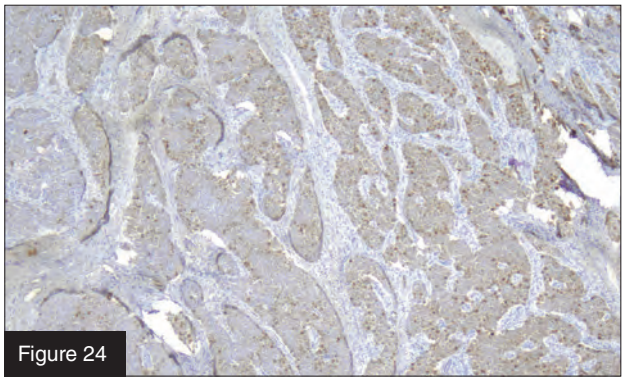


Figure 24

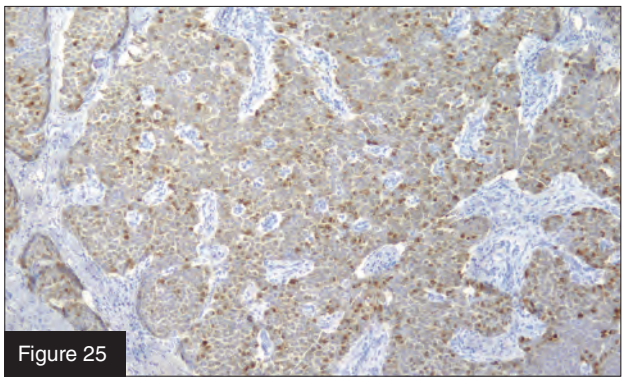


Figure 25

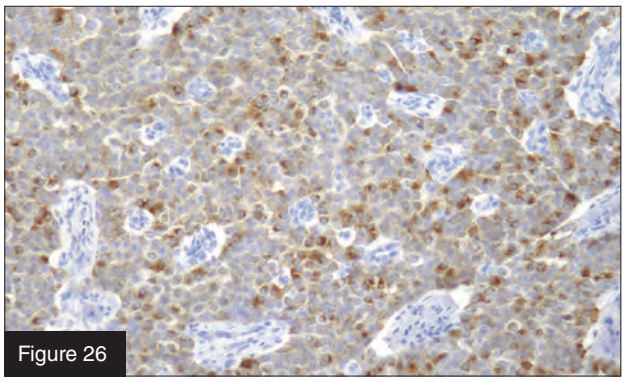


Figure 26

Figure 21: Breast carcinoma, brown staining is apparent. *(4x magnification)*.
Figure 22: In this breast carcinoma, homogeneous non-specific cytoplasmic staining can be seen. *(20x magnification)*.
Figure 23: Breast carcinoma with no membranous staining seen. *(40x magnification)*.
Figure 24: Breast carcinoma with dot artifact. *(4x magnification)*.
Figure 25: Breast carcinoma exhibits brown, dot artifact staining. *(10x magnification)*.
Figure 26: Breast carcinoma with brown dots representing cytoplasmic staining, not membrane staining. *(20x magnification)*.

Artifacts

Interpreting Artifacts

Edge Artifact

Commonly, edge artifacts are linked to the preanalytic handling of the tissue. Often the method of surgical extraction is the cause (see Crushing and Thermal artifact sections). This phenomenon is more frequently observed with the advent of stereotactic needle biopsies. This artifact occurs in 3-5% of cases.

- Inadequate processing of thick tissue samples may mimic edge artifact by rendering the central portion of the tissue sub-optimally fixed relative to the peripheral areas. In these circumstances, the immunoreactivity based on the sub-optimal central portion may be mistakenly interpreted as false-negative as optimal fixation is only present at the periphery.
- Frequently, increased staining is observed around the periphery of the tissue specimen, known as the “edge effect”.
  - The edge effect represents artifact due to tissue drying prior to fixation.
  - If the positive reaction is only at the edge of the tissue section (i.e. a few layers of staining at the periphery and ending abruptly with penetration into the centrally located tumor), grading at the edge of the tissue specimen should be avoided.

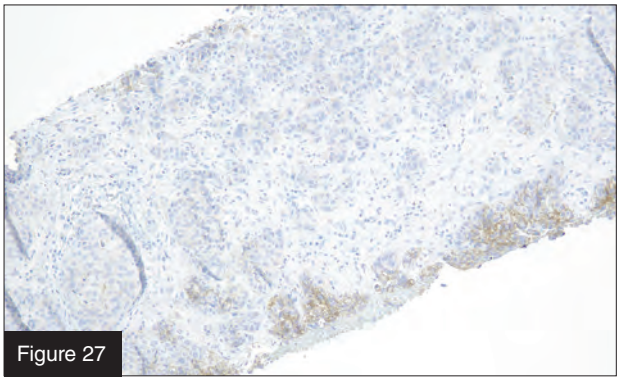


Figure 27

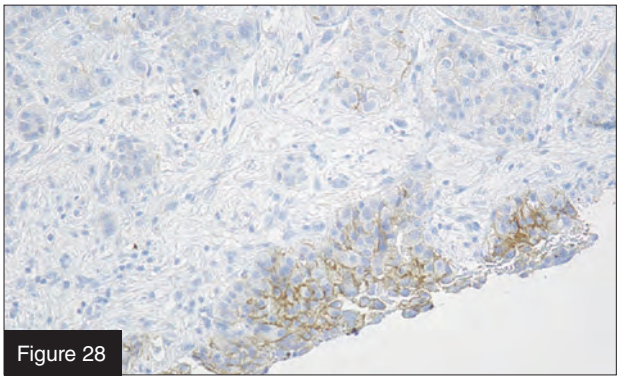


Figure 28

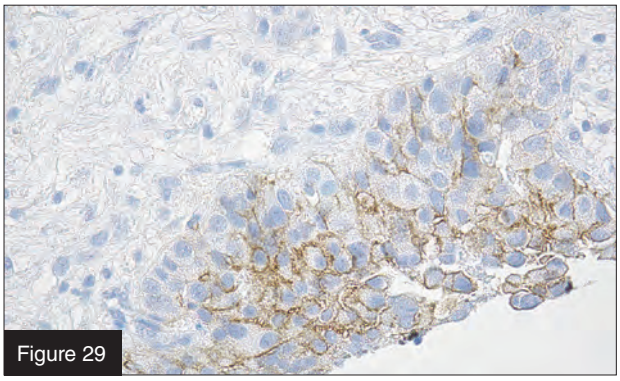


Figure 29

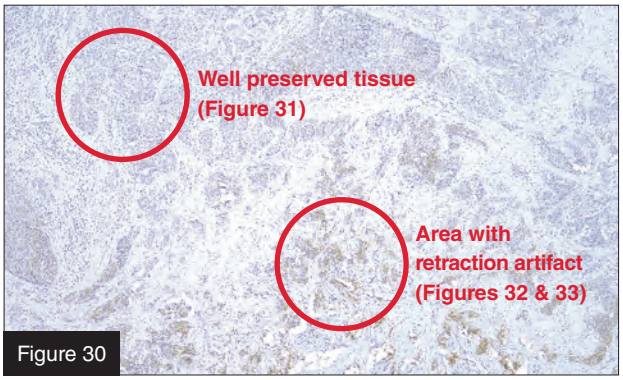
Figure 27: Breast carcinoma, edge artifact, is obvious. *(10x magnification)*.

Figure 28: Breast carcinoma, edge artifact. *(20x magnification)*.

Figure 29: Breast carcinoma, edge artifact. *(40x magnification)*.

Retraction Artifact

Retraction artifact is edge artifact on a cellular level and can be observed in diagnostic entities such as basal cell carcinoma. Unfortunately, in many infiltrating breast carcinomas, the desmoplastic status may cause retraction of the epithelial cells from the stroma. This creates small spaces where antibody and chromogen can pool around the epithelial cells forming circumferential deposition of the brown stain. This artifact requires thorough examination of the intercellular areas (i.e. cell-to-cell interfaces not the cell-to-stroma interface). Retraction artifacts occur in 2-5% of cases.



Retraction of the epithelial cells from the stroma with deposition of the chromogen circumferentially around clusters of cells but little to no immunoreactivity in the cell-to-cell interface. Score 1+

Figure 30:
Breast carcinoma. *(4x magnification)*.

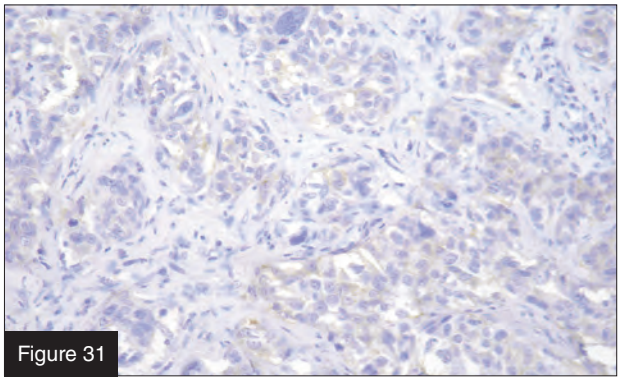


Figure 31: Immunoreactivity in the well-preserved area of breast carcinoma is 1+. *(10x magnification)*.

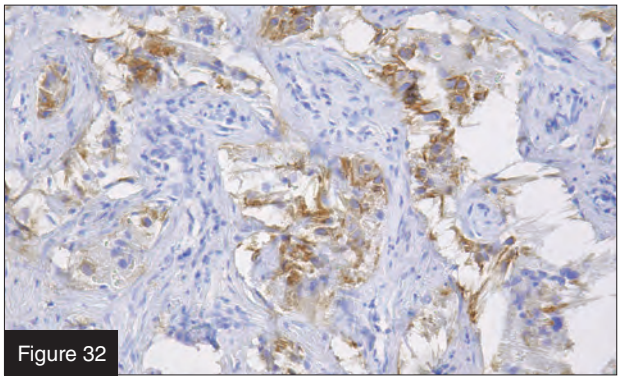


Figure 32: Breast carcinoma with non-specific immunoreactivity is apparent as retraction artifact. *(20x magnification)*.

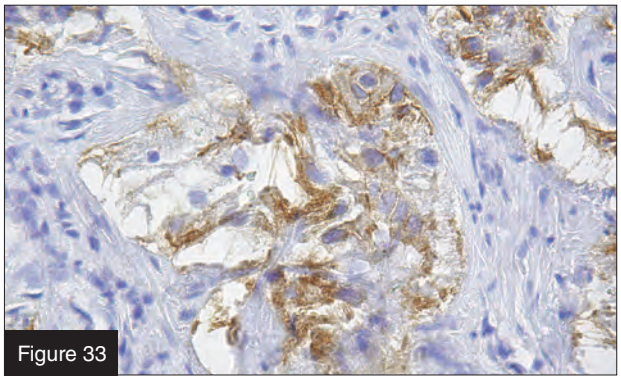


Figure 33: Breast carcinoma with non-specific immunoreactivity is confirmed. *(40x magnification)*.

Thermal Artifact

This artifact occurs at the preanalytic stage. The surgical removal of tissue with an electrocautery instrument is detrimental to the preservation of the tissue. This is especially true and inversely proportional to the size of the tissue (i.e. the smaller the biopsy the more damage incurred). The frequency is dependent upon the surgeon and his/her preferred method of tissue procurement. Thermal artifacts may occur in 3-5% of cases.

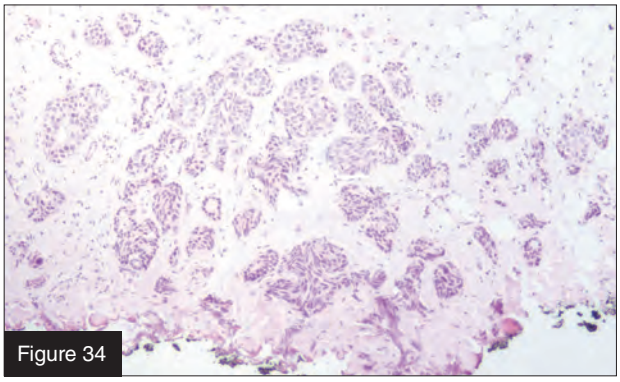


Figure 34

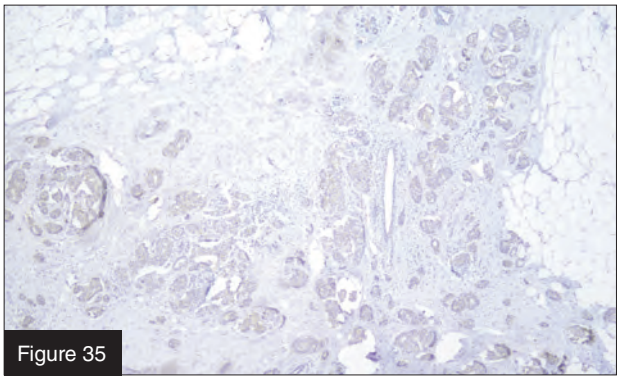


Figure 35

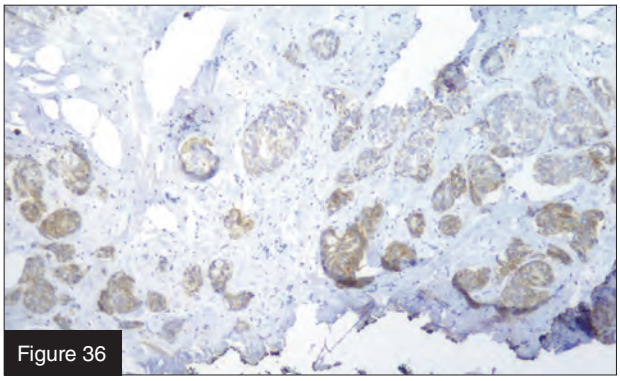


Figure 36

Example of Thermal Artifact

Figure 34: Breast carcinoma with thermal artifact can best be seen on the H&E. The majority of the injury can be seen at the edge. As heat transfers through the tissue, less and less can be seen. *(10x magnification)*.

Figure 35: Breast carcinoma with burning around the edges is slightly apparent. Central part of the lesion is the best preserved area. 1+ score is apparent. *(4x magnification)*.

Figure 36: Breast carcinoma with thermal artifact. Score: 1+ Non-specific deposition of the chromogen in a pattern consistent with specific HER2 is localized in areas with typical morphologic features of the thermal injury. The centrally located tumor is HER2 negative. *(10x magnification)*.

Crush Artifact

Crush artifact is related closely to edge artifact. This artifact may be encountered more often in stereotactic 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 tissues along the edges of the core can produce a linear staining that has to be interpreted as artifact. This artifact occurs in less than 1% of cases.

- Inadvertent crushing of the tissue occasionally occurs during sectioning resulting in morphologically distorted cellular architecture.
- When compared to surrounding cells, stronger staining may be observed in crushed cells. Crushed cells typically demonstrate condensed nuclei. Crushed cells should be avoided in grading.

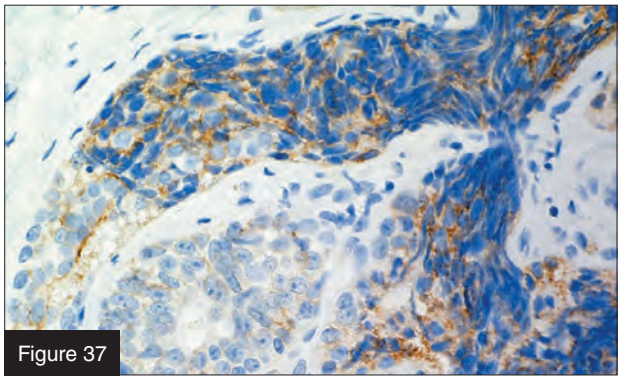


Figure 37

- Deposition of the chromogen is characteristic in areas where the cells are crushed while the central well-preserved cells are devoid of immunoreactivity.

Decalcification Artifact

The spinal vertebrae and other areas of the human skeleton are sites of metastatic carcinoma. Interventional radiology has facilitated access to domains of the body and has provided another source of specimens that can be tested for analytes such as HER2. However, in order to render the tissue soft enough to cut on a histologist's microtome (at 4-5 microns) the tissue has to be decalcified. This traditionally is accomplished by exposing the bony tissue to a variety of available decalcification solutions. This renders the tissue soft enough to obtain good histologic section but also renders the tissue less than optimal for immunostains. The use of HercepTest™ on decalcified tissues has not been validated and is not recommended.

Carcinoma has darker staining on crushed areas. Score 1+

Figure 37: Breast carcinoma showing crush artifact. *(40x magnification).*

Effects of Fixation

Standardization of fixation is very important when using HercepTest™. These stains have been fixed for 18-24 hours and for one week, respectively.

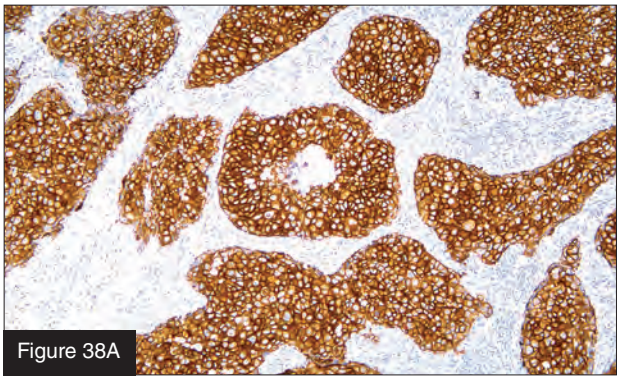


Figure 38A

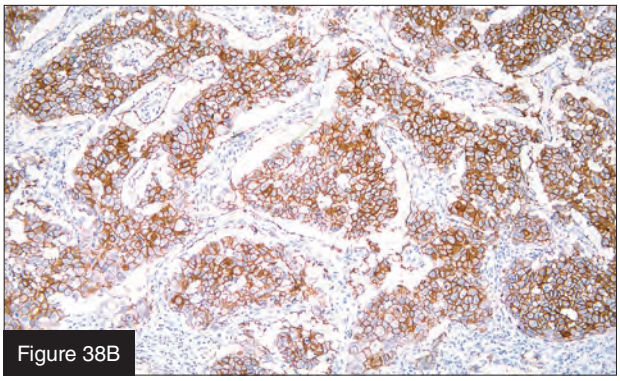


Figure 38B

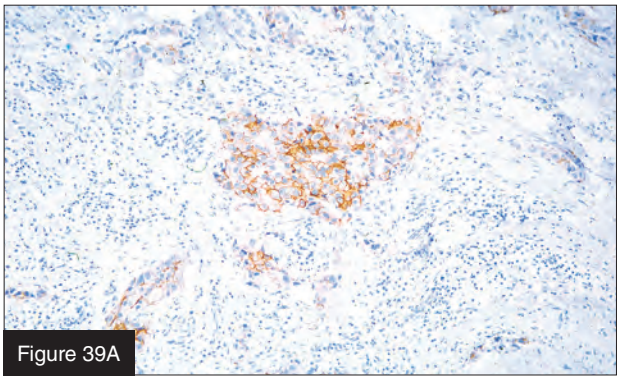


Figure 39A

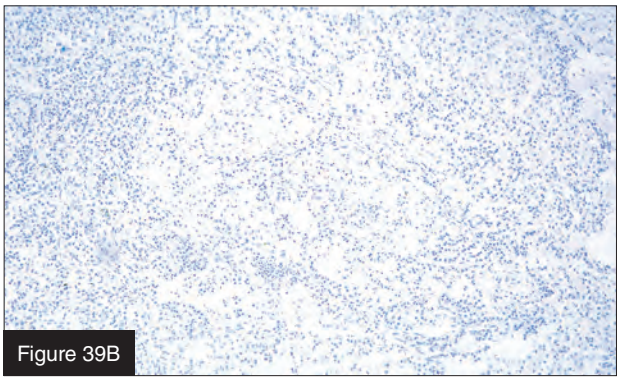


Figure 39B

Figure 38A: Breast carcinoma shows a strong 3+ staining with the appropriate fixation time.

Figure 38B: Breast carcinoma shows a noticeably weaker staining, but still 3+ after the extended fixation.

Figure 39A: Breast carcinoma shows 2+ staining with the appropriate fixation time.

Figure 39B: Breast carcinoma shows negative staining after the extended fixation.

Effects of Insufficient Target Retrieval

It is important to adhere to the target retrieval procedure described in the Instructions for Use for HercepTest™. The stains displayed to the left are sections from the same tissue, but exposed to appropriate epitope retrieval and insufficient epitope retrieval, respectively.

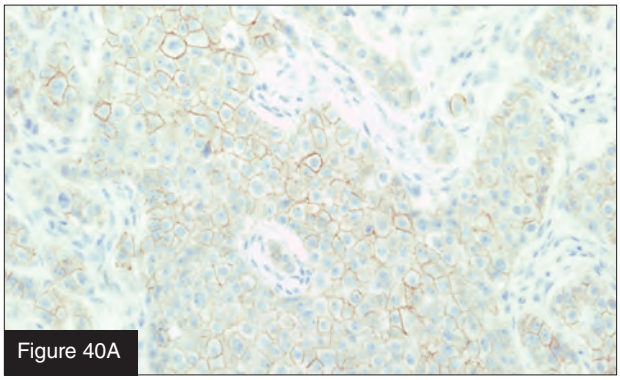


Figure 40A

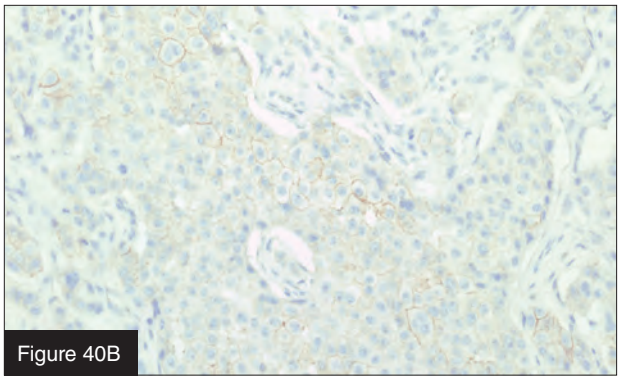


Figure 40B

Figure 40A: Breast carcinoma displaying a 2+ staining when appropriate epitope retrieval is used (40 min at 95-99 °C). *(20x magnification)*.

Figure 40B : Breast carcinoma displaying a 1+ staining when insufficient epitope retrieval is used (20 min at 90 °C). *(20x magnification)*.

Effects of Excessive Tissue Drying

Loss of Specific Staining

Excessive heating for more than 1 hour at ≥ 60 °C may cause a significant decrease or loss of the specific membrane-associated HER2 immunoreactivity.

The decreased HER2 immunostaining is likely caused by the destruction of the epitope(s) recognized by the HER2 antibodies.

- Use proper procedure for tissue drying: The drying temperature should be 60 °C for a maximum of 1 hour, 37 °C overnight, or room temperature for 12 hours or longer.
- Use validated equipment (oven, thermometer) when conducting the tissue drying.

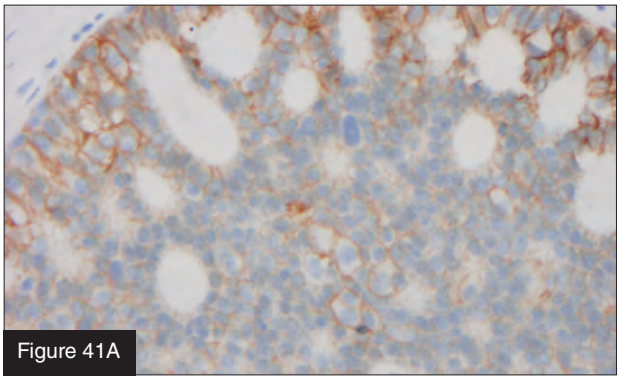


Figure 41A

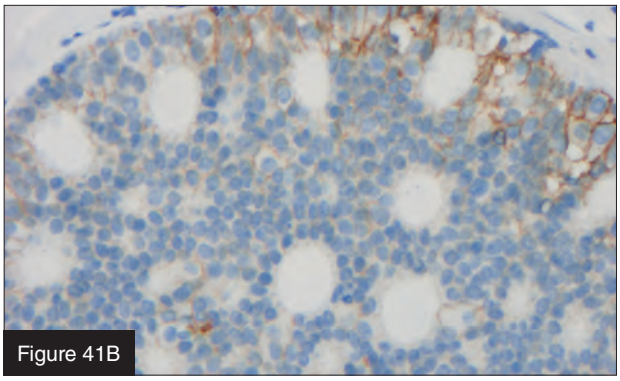


Figure 41B

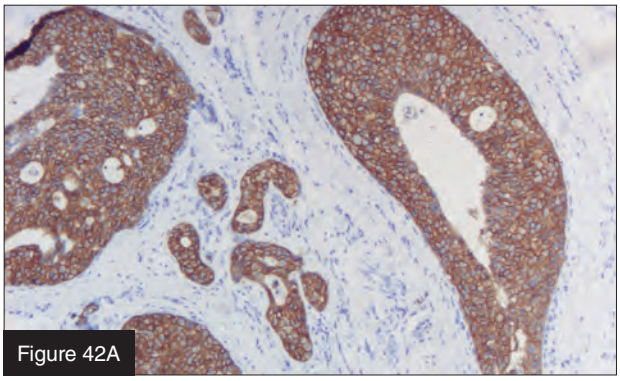


Figure 42A

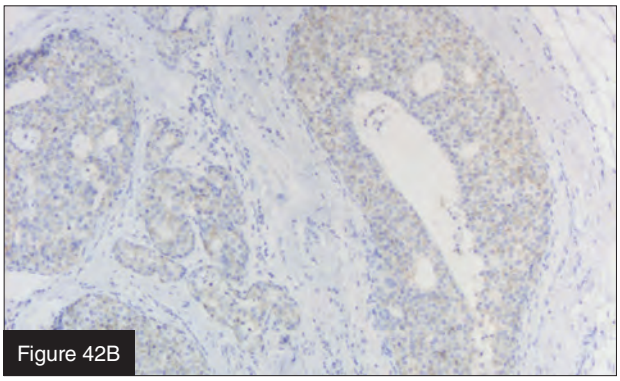


Figure 42B

Figure 41A: Breast carcinoma displaying a 2+ staining after appropriate tissue drying.

Figure 41B: Breast carcinoma displaying a noticeably weaker 1+ staining after excessive tissue drying.

Figure 42A: Breast carcinoma displaying a strong 3+ staining after appropriate tissue drying.

Figure 42B: Breast carcinoma displaying a negative staining after excessive tissue drying.

Staining Images

HER2 Expression in Various Diagnostic Entities

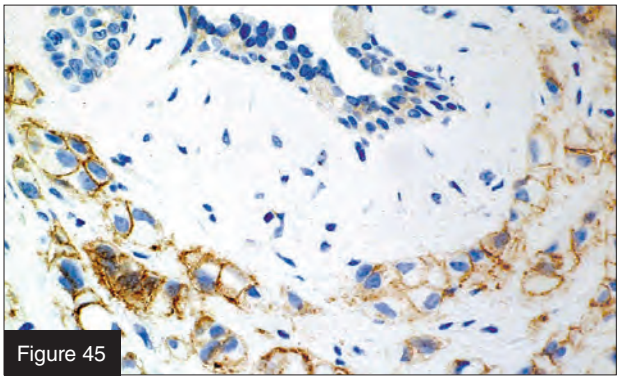


Figure 45

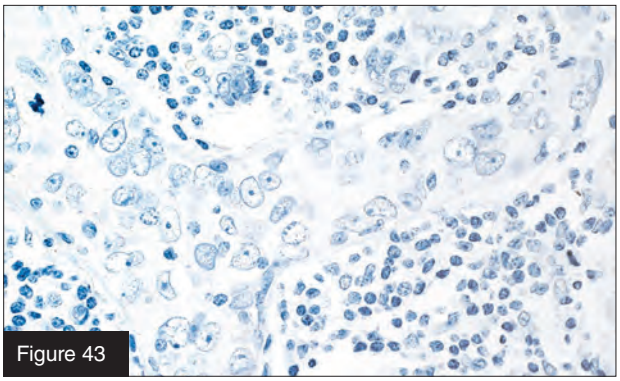


Figure 43

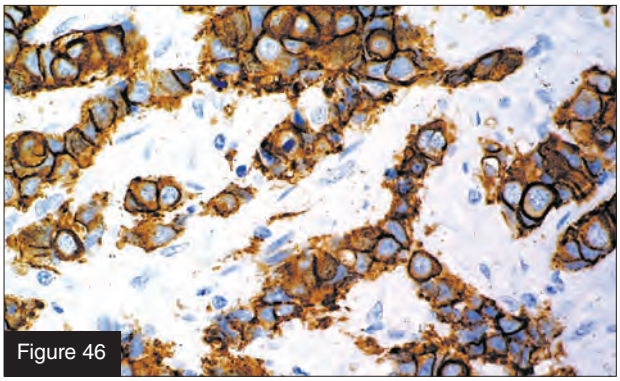


Figure 46

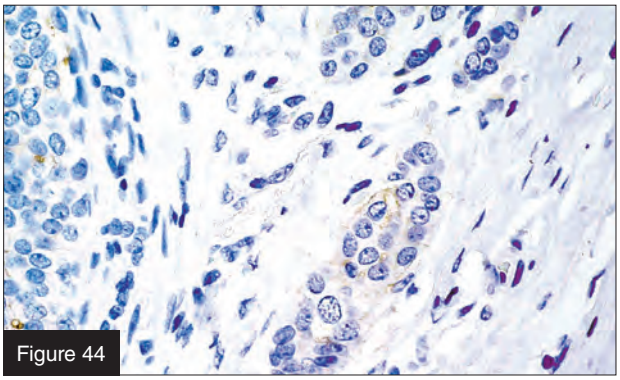


Figure 44

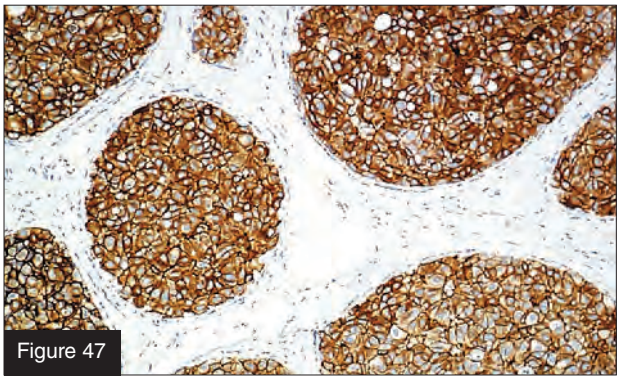


Figure 47

Figure 43: Example of poorly differentiated ductal carcinoma. Score 0 *(40x magnification)*.

Figure 44: Example of well differentiated ductal carcinoma. Score 1+ *(40x magnification)*.

Figure 45: Example of moderately differentiated ductal carcinoma. Score 2+ *(40x magnification)*.

Figure 46: Example of poorly differentiated ductal carcinoma. Score 3+ *(40x magnification)*.

Figure 47: Example of intraductal carcinoma (DCIS). Score 0 *(40x magnification)*.

HercepTest™ Score 0

No staining is seen in this invasive ductal carcinoma.

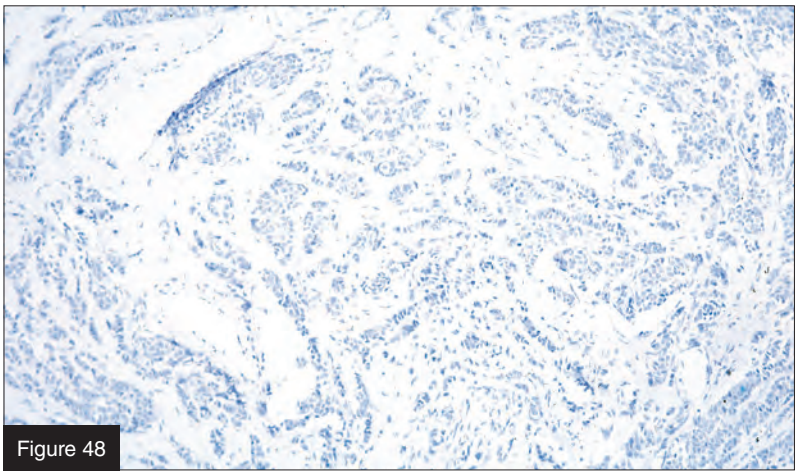


Figure 48

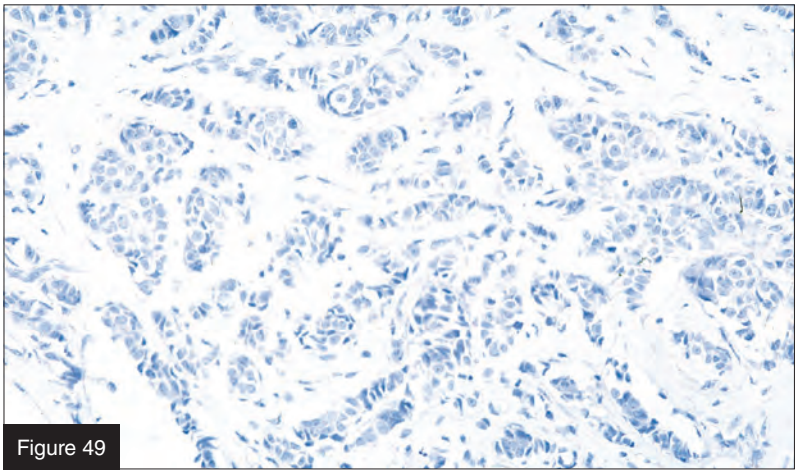


Figure 49

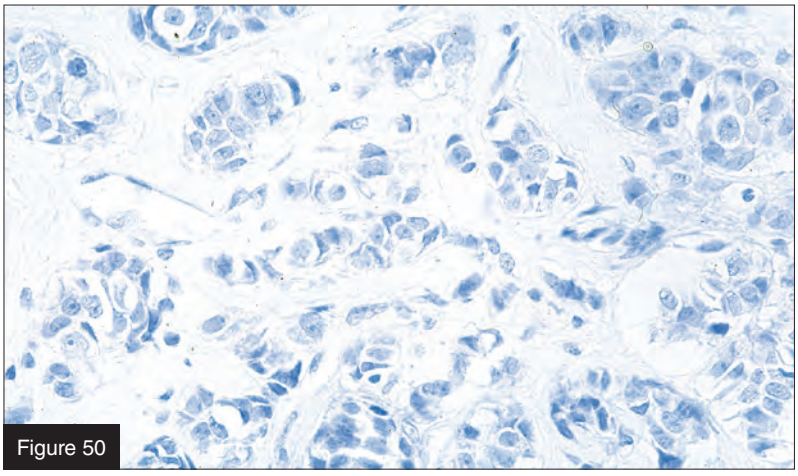


Figure 50

Figure 48: Breast carcinoma. Score 0 *(4x magnification)*.

Figure 49: Breast carcinoma. Score 0 *(10x magnification)*.

Figure 50: Breast carcinoma. Score 0 *(20x magnification)*.

HercepTest™ Score 1+

The infiltrating tumor cells are weakly stained and do not demonstrate complete membrane staining.

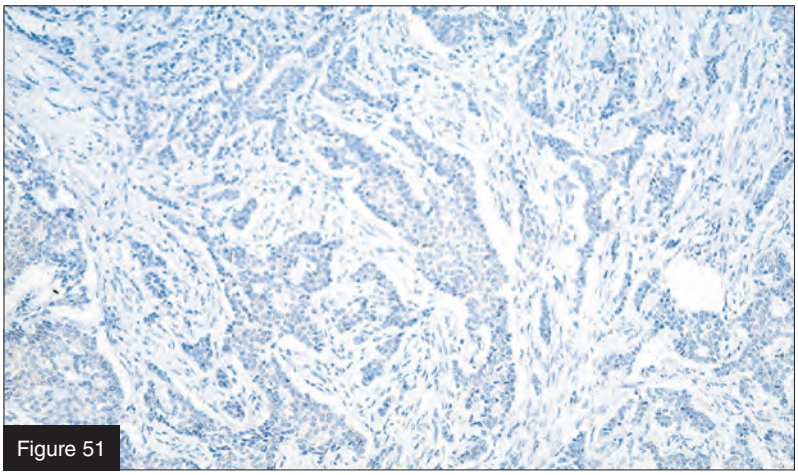


Figure 51

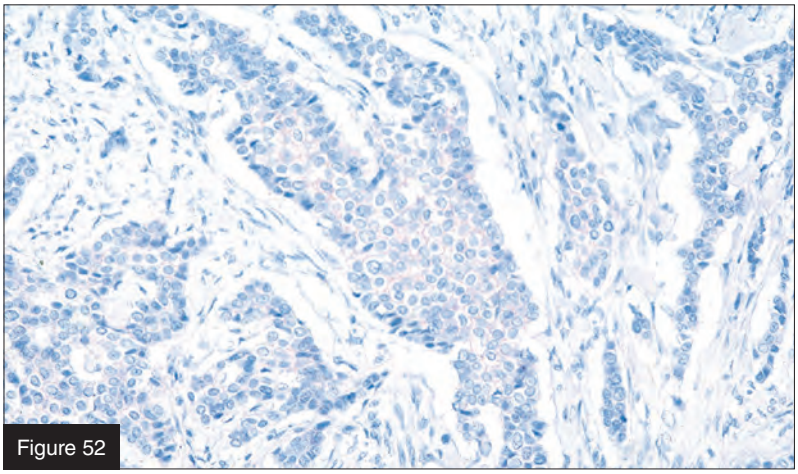


Figure 52

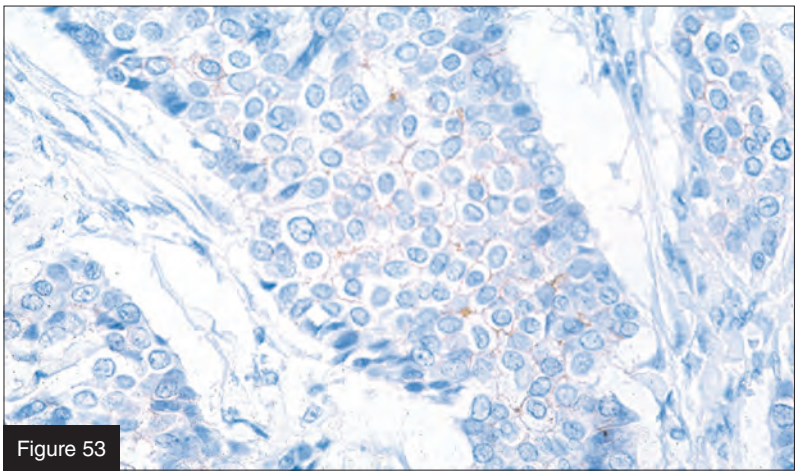


Figure 53

Figure 51: Breast carcinoma. Score 1+ *(4x magnification)*.

Figure 52: Breast carcinoma. Score 1+ *(10x magnification)*.

Figure 53: Breast carcinoma. Score 1+ *(20x magnification)*.

HercepTest™ Score 1+

The infiltrating tumor cells are weakly stained and do not demonstrate complete membrane staining.

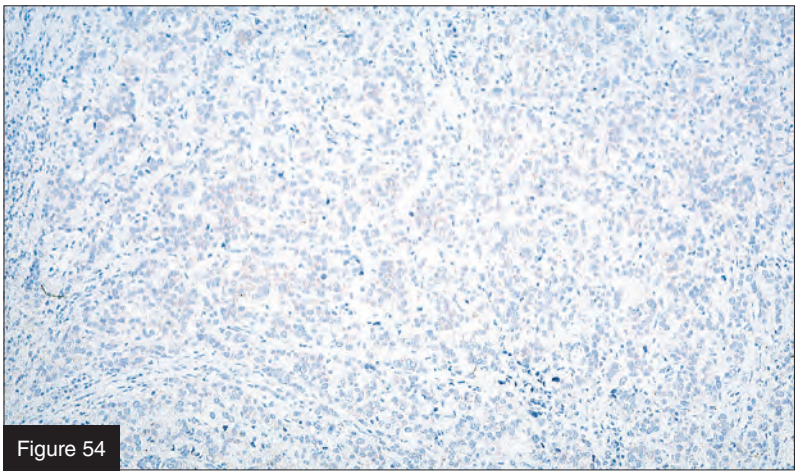


Figure 54

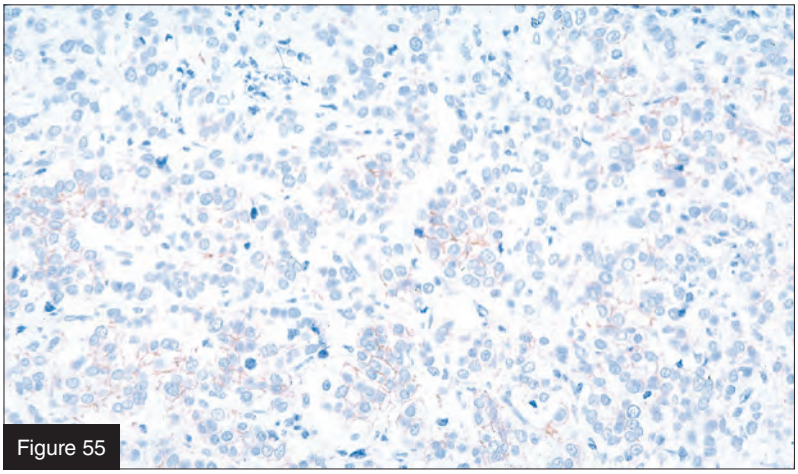


Figure 55

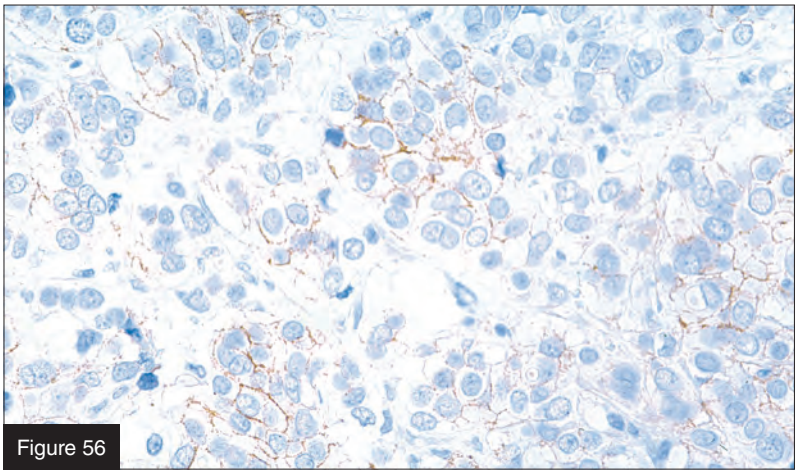


Figure 56

Figure 54: Breast carcinoma. Score 1+ *(4x magnification)*.

Figure 55: Breast carcinoma. Score 1+ *(10x magnification)*.

Figure 56: Breast carcinoma. Score 1+ *(20x magnification)*.

HercepTest™ Score 1+

The infiltrating tumor cells are weakly stained and do not demonstrate complete membrane staining.

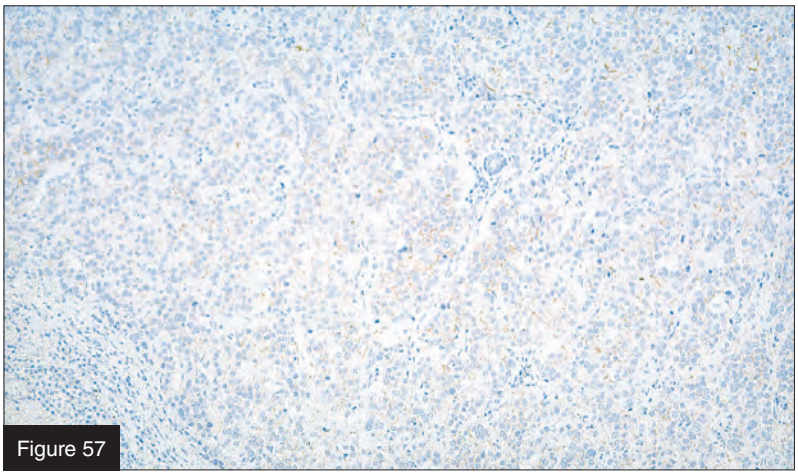


Figure 57

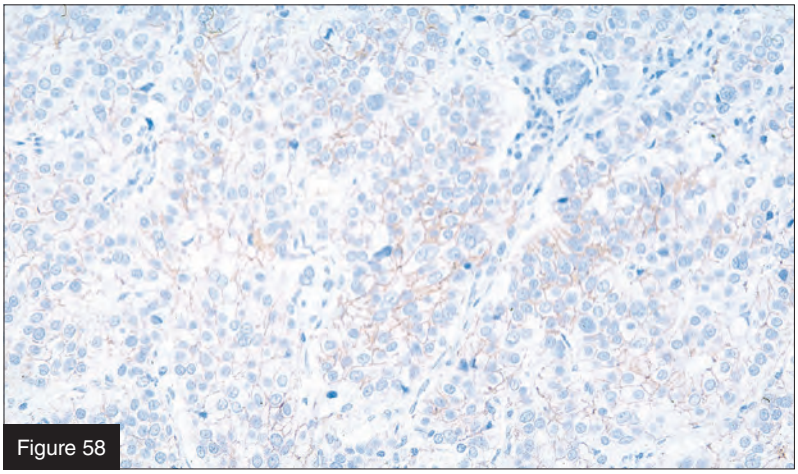


Figure 58

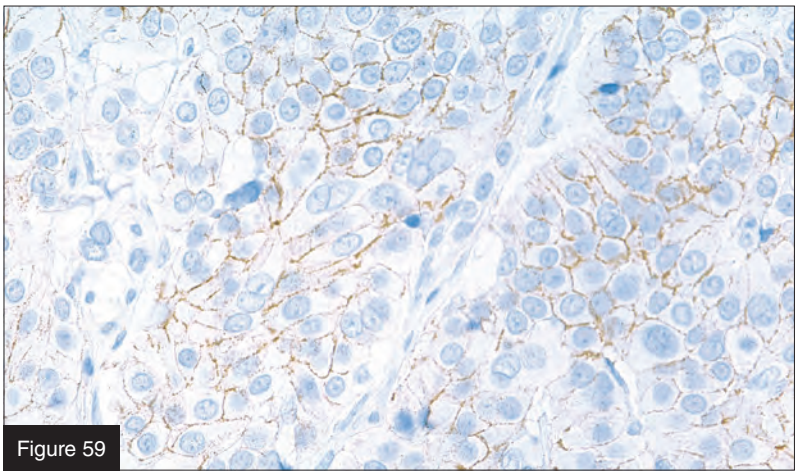


Figure 59

Figure 57: Breast carcinoma. Score 1+ *(4x magnification)*.

Figure 58: Breast carcinoma. Score 1+ *(10x magnification)*.

Figure 59: Breast carcinoma. Score 1+ *(20x magnification)*.

HercepTest™ Score 1+

The infiltrating tumor cells are weakly stained and do not demonstrate complete membrane staining.

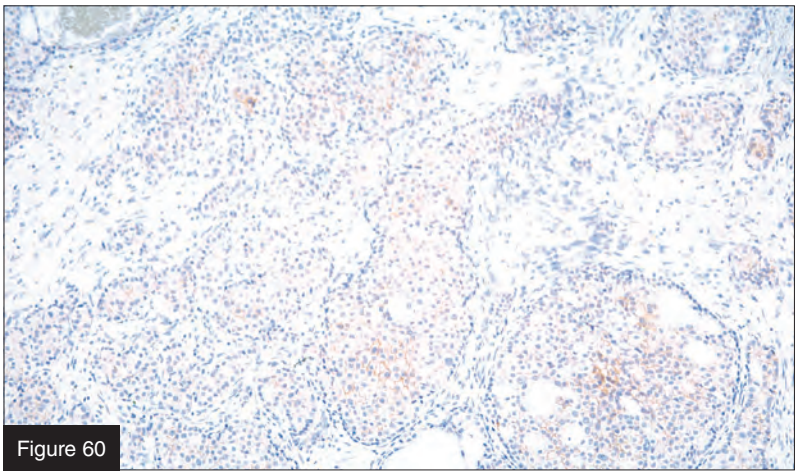


Figure 60

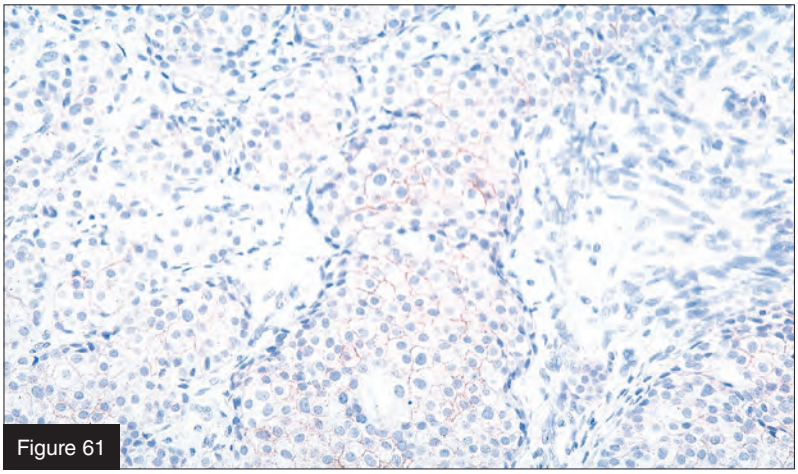


Figure 61

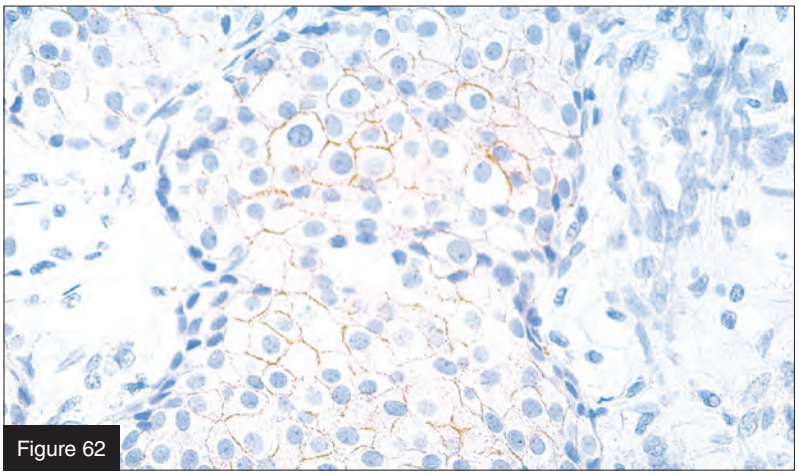


Figure 62

Figure 60: Breast carcinoma. Score 1+ *(4x magnification)*.

Figure 61: Breast carcinoma. Score 1+ *(10x magnification)*.

Figure 62: Breast carcinoma. Score 1+ *(20x magnification)*.

HercepTest™ Score 2+

These infiltrating tumor cells exhibit complete membrane staining; the intensity is moderate.

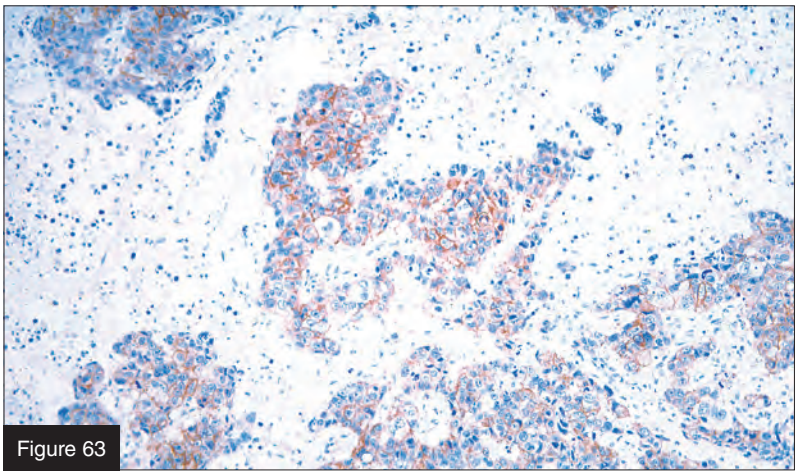


Figure 63

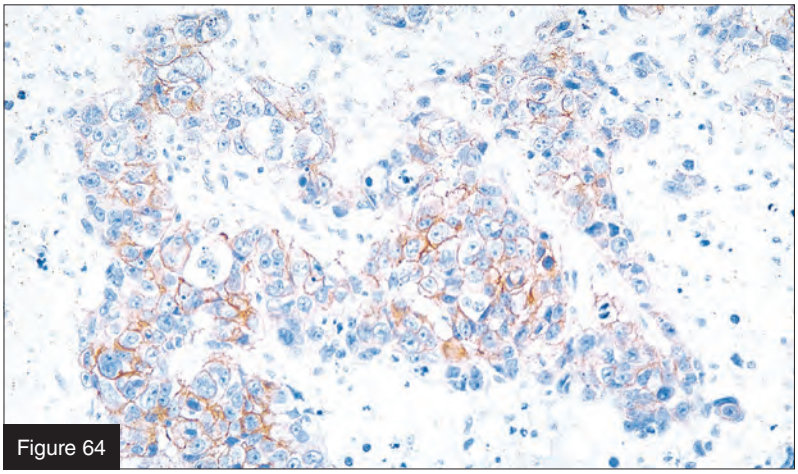


Figure 64

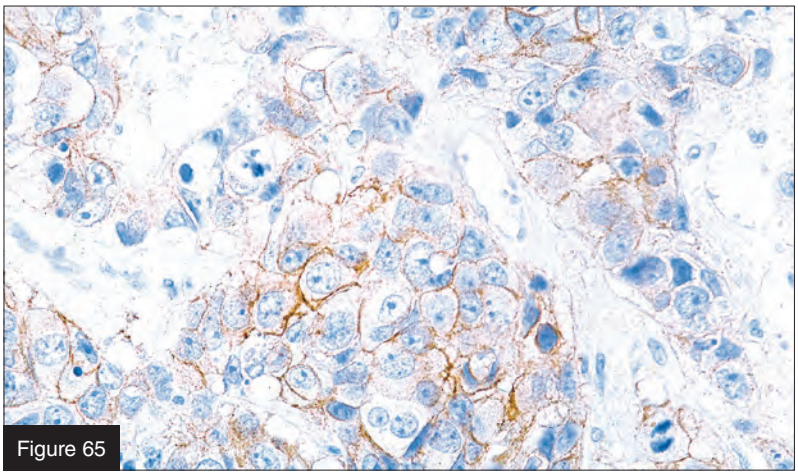


Figure 65

Figure 63: Breast carcinoma. Score 2+ *(4x magnification)*.

Figure 64: Breast carcinoma. Score 2+ *(10x magnification)*.

Figure 65: Breast carcinoma. Score 2+ *(20x magnification)*.

HercepTest™ Score 2+

These infiltrating tumor cells exhibit complete membrane staining; the intensity is moderate.

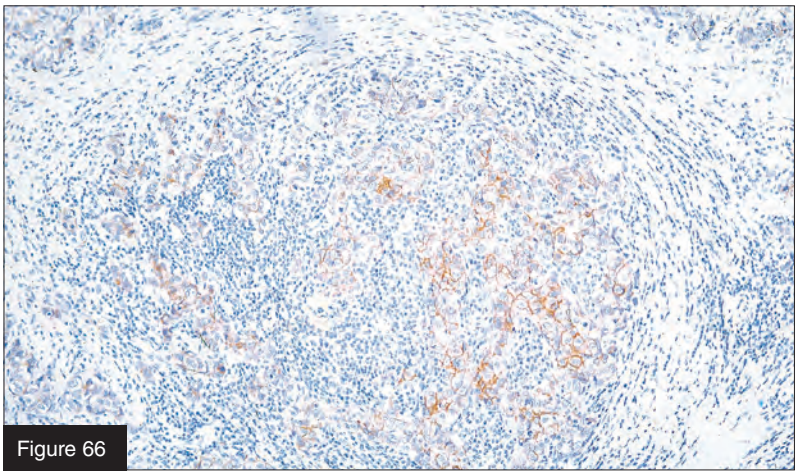


Figure 66

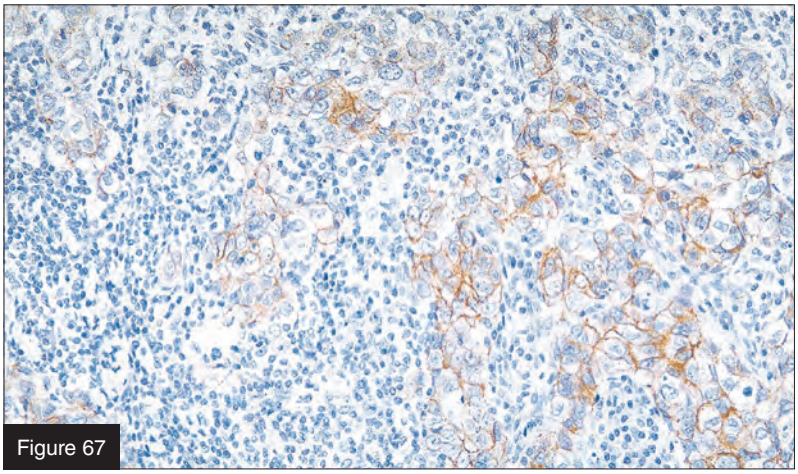


Figure 67

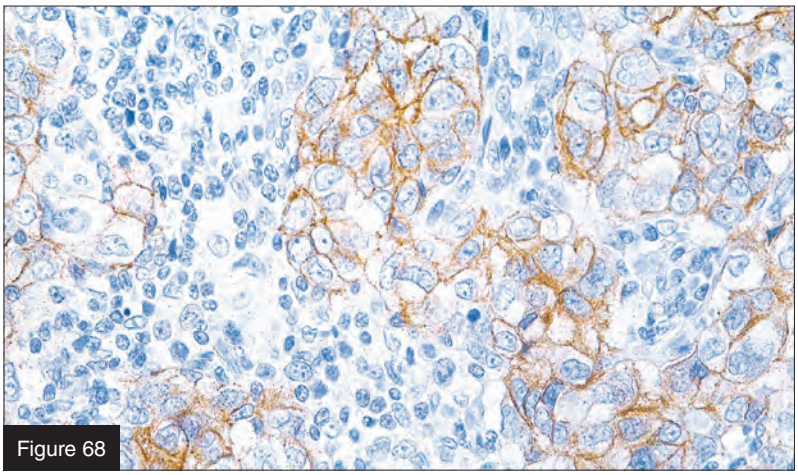


Figure 68

Figure 66: Breast carcinoma. Score 2+ *(4x magnification)*.

Figure 67: Breast carcinoma. Score 2+ *(10x magnification)*.

Figure 68: Breast carcinoma. Score 2+ *(20x magnification)*.

HercepTest™ Score 2+

These infiltrating tumor cells exhibit complete membrane staining; the intensity is moderate.

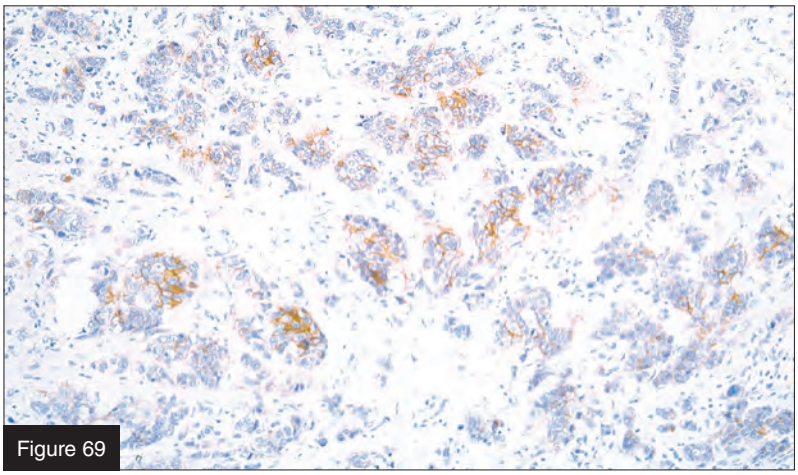


Figure 69

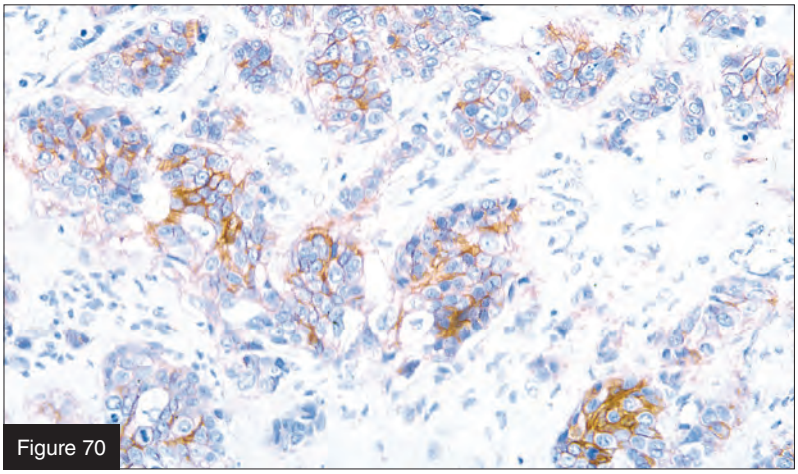


Figure 70

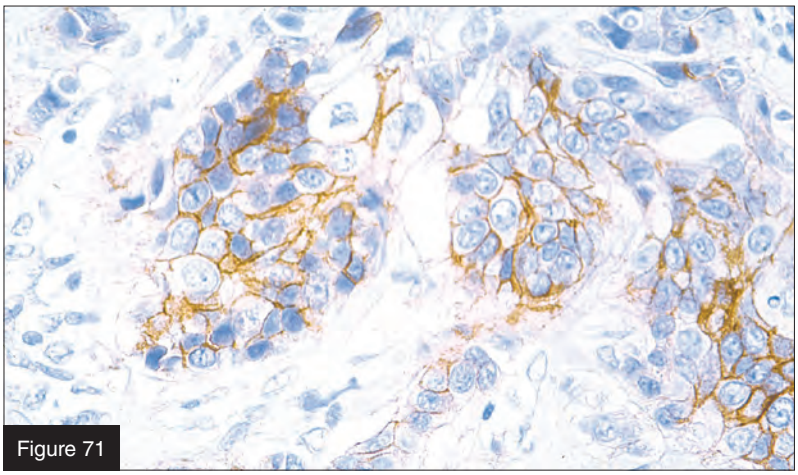


Figure 71

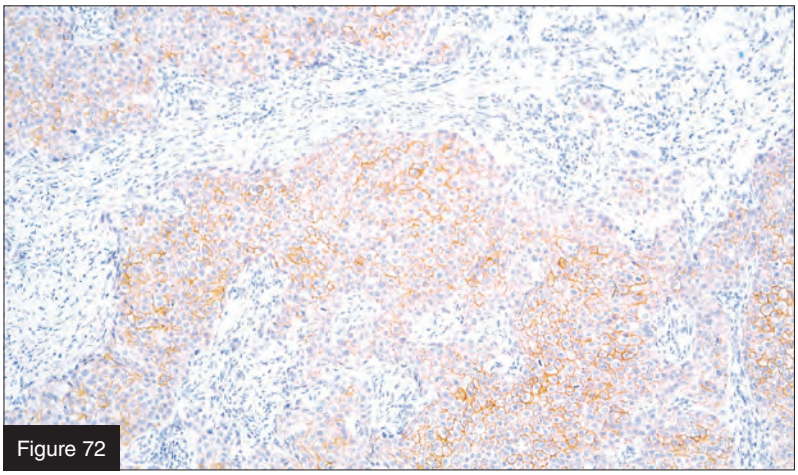
Figure 69: Breast carcinoma. Score 2+ *(4x magnification)*.

Figure 70: Breast carcinoma. Score 2+ *(10x magnification)*.

Figure 71: Breast carcinoma. Score 2+ *(20x magnification)*.

HercepTest™ Score 2+

These infiltrating tumor cells exhibit complete membrane staining; the intensity is moderate.



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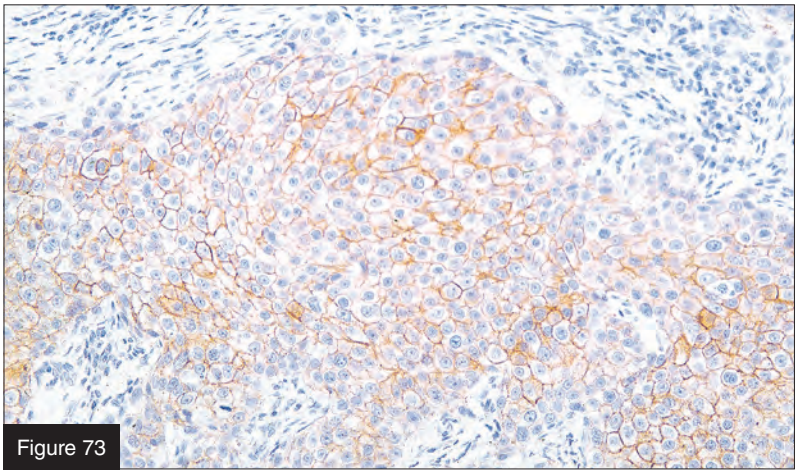


Figure 73

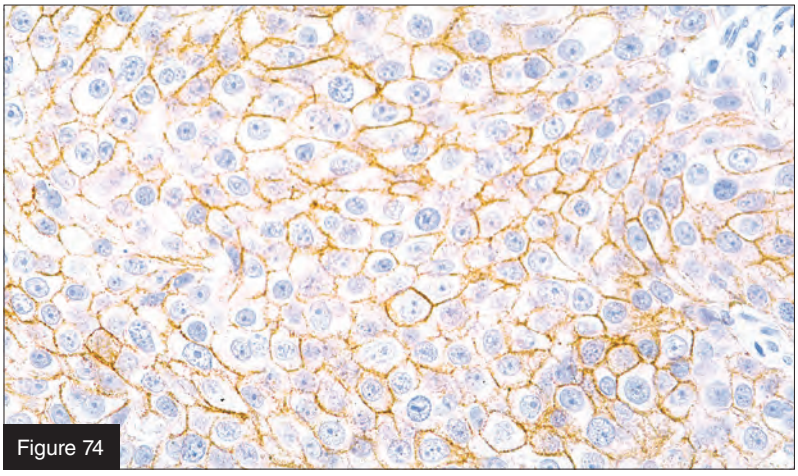


Figure 74

Figure 72: Breast carcinoma. Score 2+ *(4x magnification)*.

Figure 73: Breast carcinoma. Score 2+ *(10x magnification)*.

Figure 74: Breast carcinoma. Score 2+ *(20x magnification)*.

HercepTest™ Score 2+

These infiltrating tumor cells exhibit complete membrane staining; the intensity is moderate.

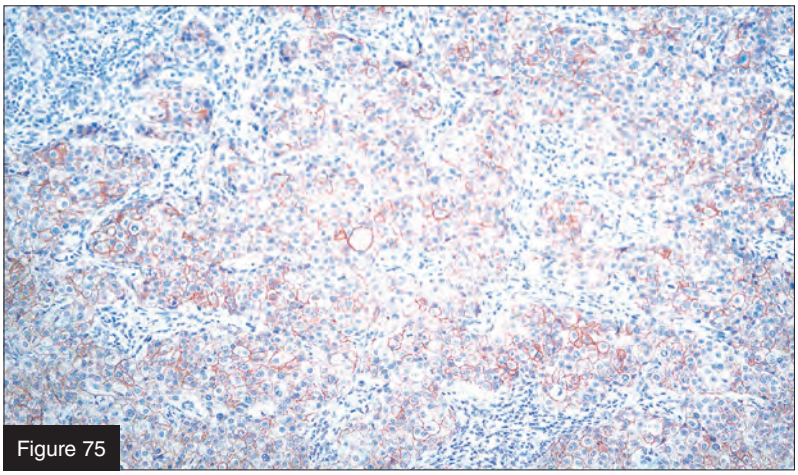


Figure 75

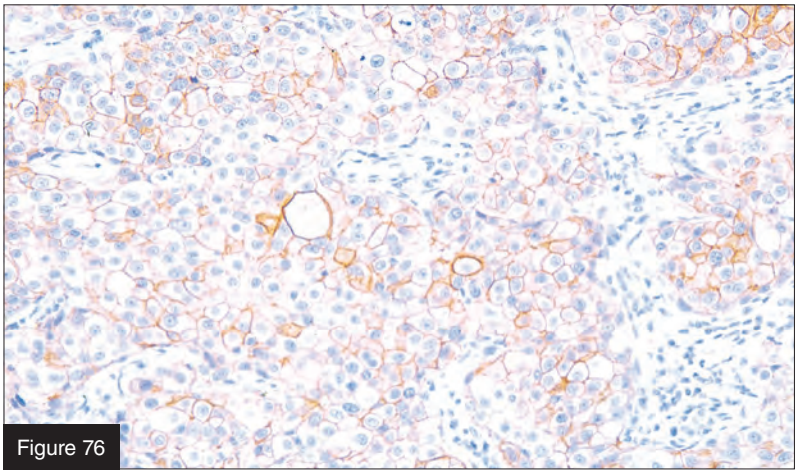


Figure 76

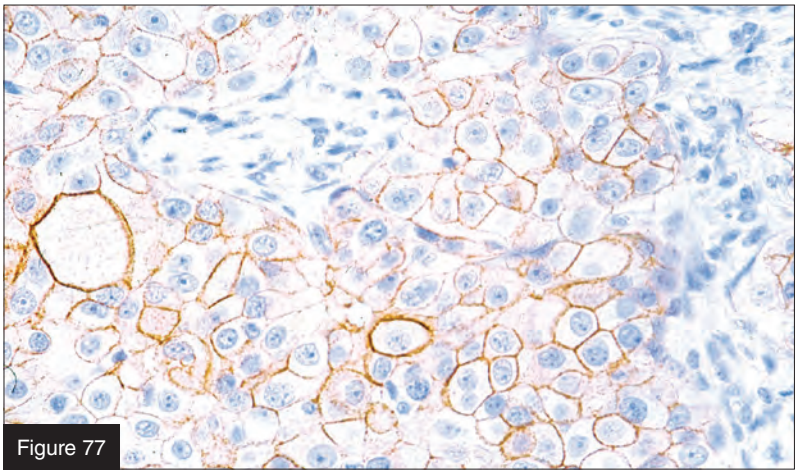


Figure 77

Figure 75: Breast carcinoma. Score 2+ *(4x magnification)*.

Figure 76: Breast carcinoma. Score 2+ *(10x magnification)*.

Figure 77: Breast carcinoma. Score 2+ *(20x magnification)*.

HercepTest™ Score 3+

The majority of infiltrating tumor cells exhibit intense, complete membrane staining.

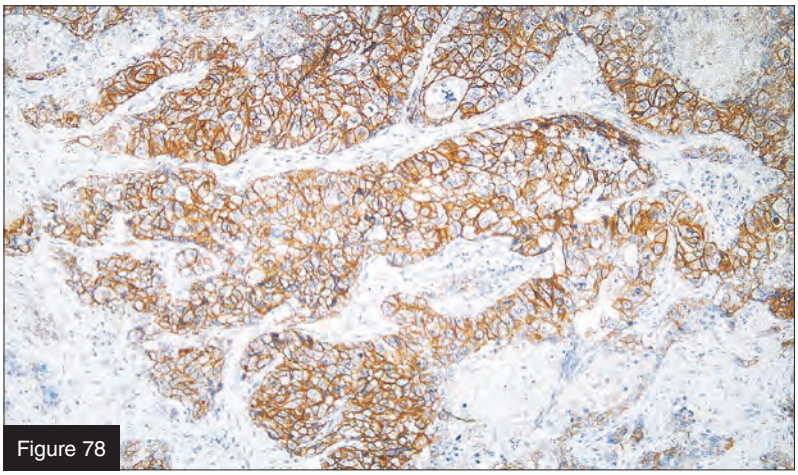


Figure 78

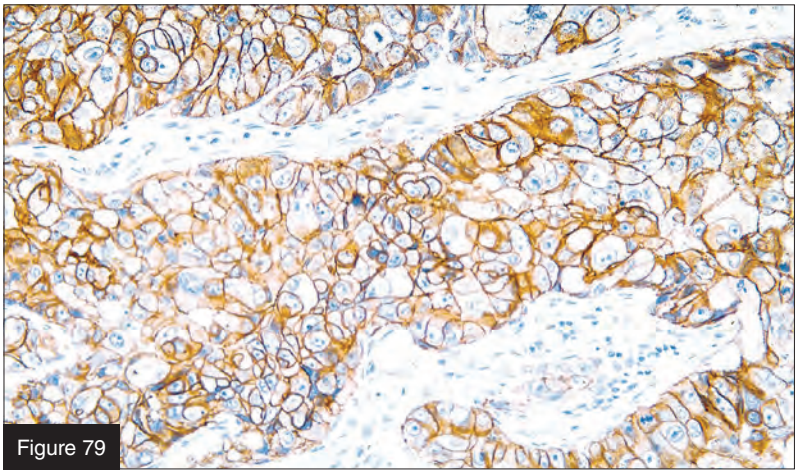


Figure 79

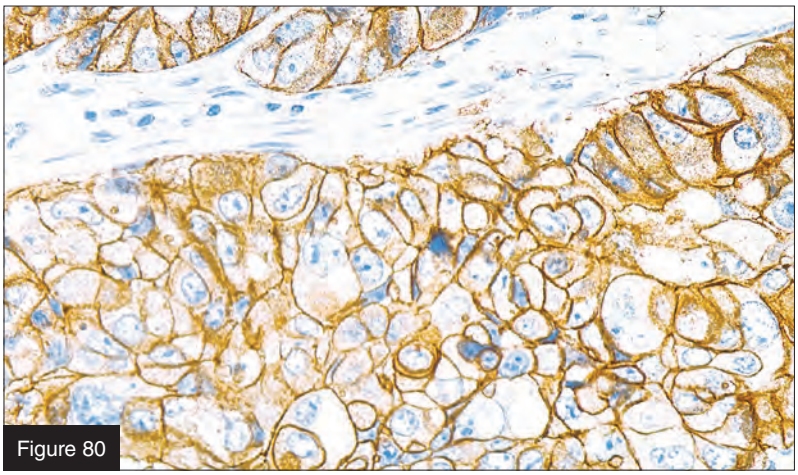


Figure 80

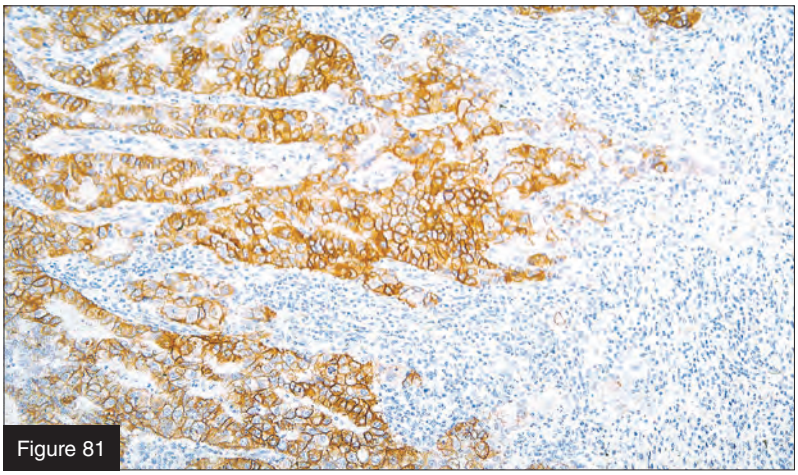
Figure 78: Breast carcinoma. Score 3+ *(4x magnification)*.

Figure 79: Breast carcinoma. Score 3+ *(10x magnification)*.

Figure 80: Breast carcinoma. Score 3+ *(20x magnification)*.

HercepTest™ Score 3+

The majority of infiltrating tumor cells exhibit intense, complete membrane staining.



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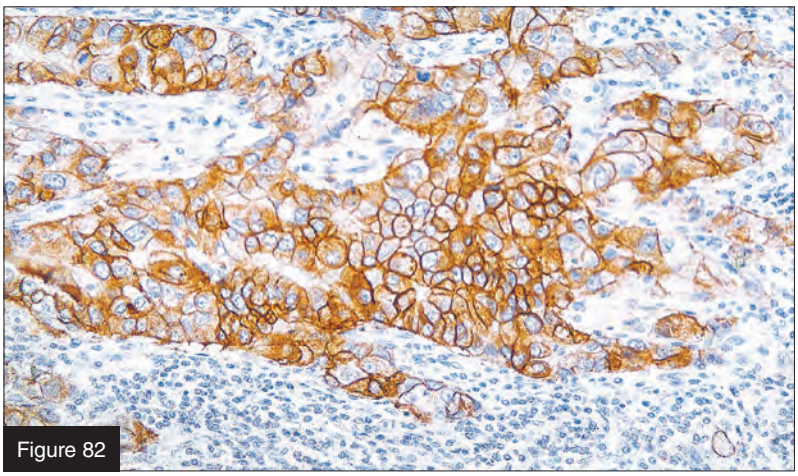

Figure 82

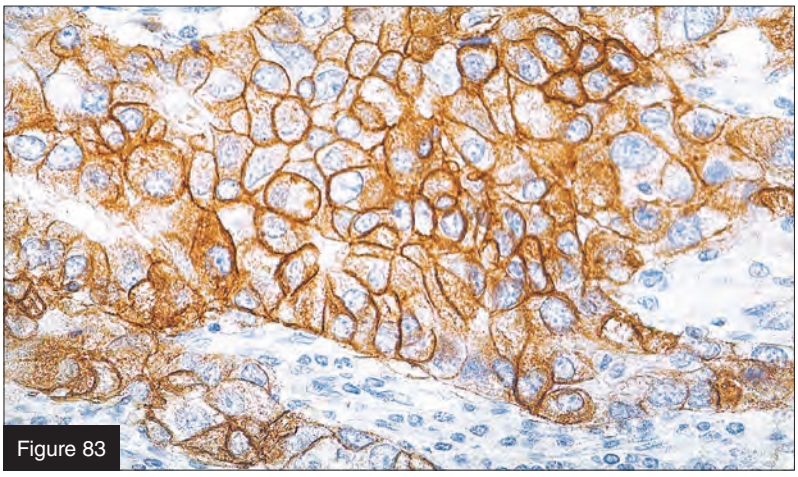

Figure 83

Figure 81: Breast carcinoma. Score 3+ *(4x magnification)*.

Figure 82: Breast carcinoma. Score 3+ *(10x magnification)*.

Figure 83: Breast carcinoma. Score 3+ *(20x magnification)*.

HercepTest™ Score 3+

The majority of infiltrating tumor cells exhibit intense, complete membrane staining.

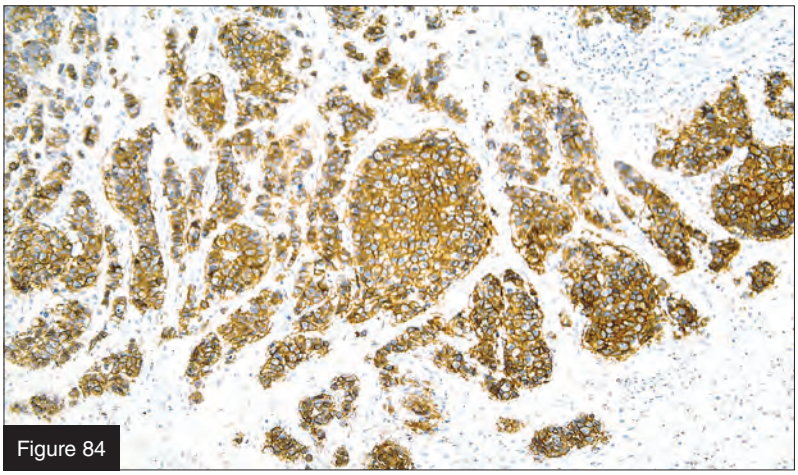

Figure 84

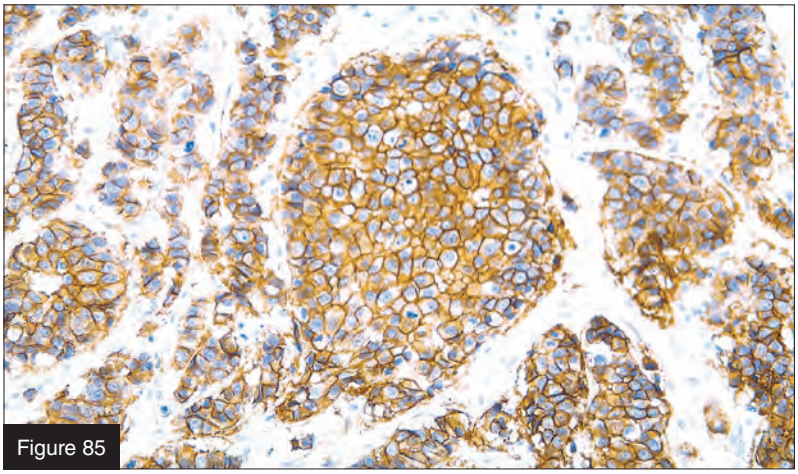

Figure 85

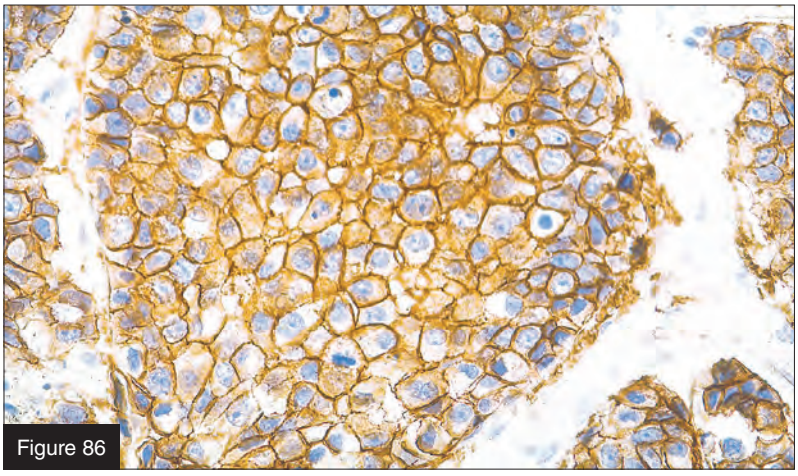

Figure 86

Figure 84: Breast carcinoma. Score 3+ *(4x magnification)*.

Figure 85: Breast carcinoma. Score 3+ *(10x magnification)*.

Figure 86: Breast carcinoma. Score 3+ *(20x magnification)*.

HercepTest™ Score 3+

The majority of infiltrating tumor cells exhibit intense, complete membrane staining.

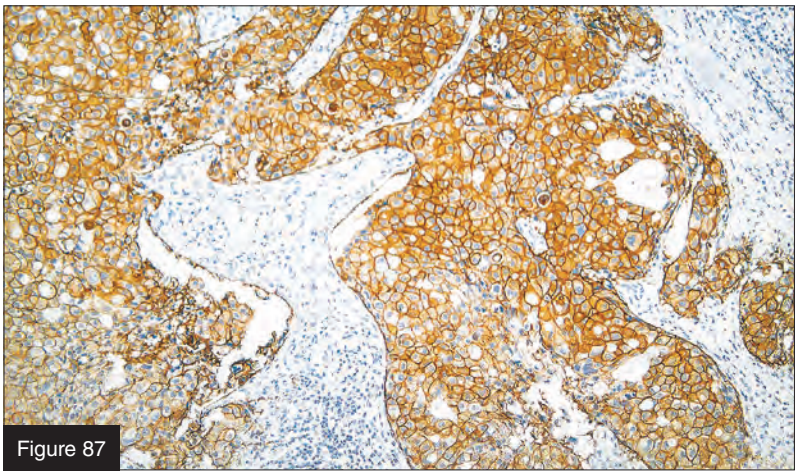

Figure 87

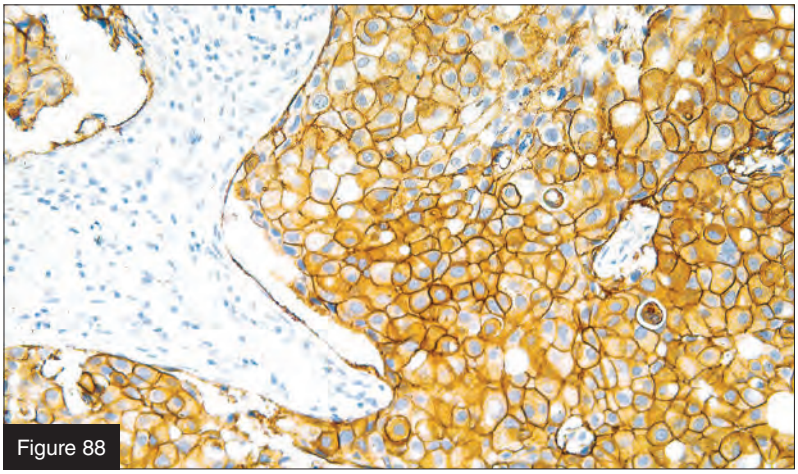

Figure 88

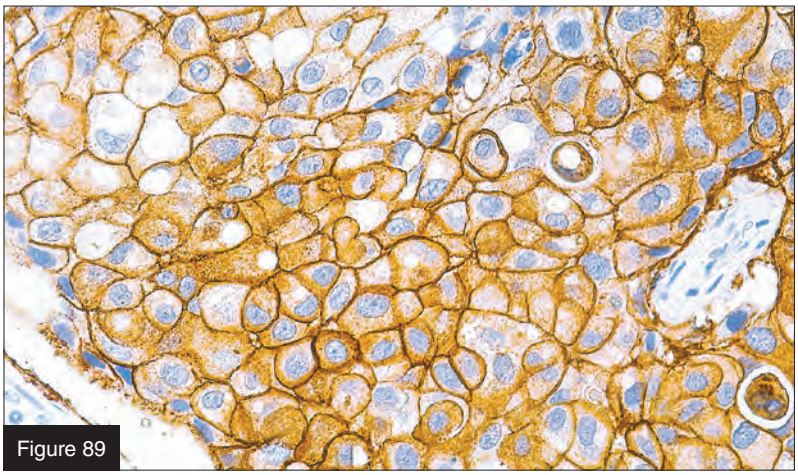

Figure 89

Figure 87: Breast carcinoma. Score 3+ *(4x magnification)*.

Figure 88: Breast carcinoma. Score 3+ *(10x magnification)*.

Figure 89: Breast carcinoma. Score 3+ *(20x magnification)*.

Troubleshooting Guide

Troubleshooting Guideline for HercepTest™

Problem	Probable Cause	Suggested Action	Reference
1. No staining of slides	1a. Programming error. Reagents not used in proper order.	Check programming grid to verify that the staining run was programmed correctly.	
	1b. Reagent vials were not loaded in the correct locations in the reagent racks.	Check the Reagent Map to verify the proper location of reagent vials.	
	1c. Insufficient reagent on tissue section.	Ensure that enough reagent is loaded into the reagent vials prior to commencing the run. Refer to the Reagent Map for volumes required. Ensure that spreading of reagent is optimal.	HercepTest™ Interpretation Manual Artificial Heterogeneous Staining (page 20)
	1d. Sodium azide in Wash Solution.	Use fresh preparation of Wash Buffer provided in the kit.	
	1e. Excessive heating for more than one hour at ≥ 60 °C may cause a significant decrease or loss of the specific membrane-associatec HER2 immunoreactivity.	Air dry the tissue sections at room temperature for a minimum of 12 hours or until dry. Alternatively, dry at 37 °C overnight or dry at 60 °C for a maximum of one hour. Drying of tissue sections at elevated temperatures must only be performed in a calibrated oven with uniform heat distribution.	HercepTest™ Interpretation Manual Excessive Tissue Drying. Loss of specific staining (page 30)
2. Weak staining of slides	2a. Inadequate epitope retrieval.	Verify that Epitope Retrieval Solution reaches 95-99 °C for full 40 minutes and is allowed to cool for an additional 20 minutes.	HercepTest™ Interpretation Manual Effects of Insufficient Target Retrieval. (page 29)
	2b. Inadequate reagent incubation times.	Review Staining Procedure instructions.	See Instructions for Use
	2c. Inappropriate fixation method used.	Ensure that patient tissue is not over-fixed or that an alternative fixative was not used.	HercepTest™ Interpretation Manual Effects of Fixation. (page 28)
	2d. Excessive heating for more than one hour at ≥ 60 °C may cause a significant decrease or loss of the specific membrane-associated HER2 immunoreactivity.	Air dry the tissue sections at room temperature for a minimum of 12 hours or until dry. Alternatively, dry at 37 °C overnight or at 60 °C for a maximum of one hour. Drying of tissue sections at elevated temperatures must only be performed in a calibrated oven with uniform heat distribution.	HercepTest™ Interpretation Manual Effects of Excessive Tissue Drying. Loss of specific staining (page 30)

Problem	Probable Cause	Suggested Action	Reference
3. Excessive background staining of slides.	3a. Paraffin incompletely removed.	Use fresh clearing solutions and follow procedure as outlined in i Instructions for Use, section B.1	HercepTest™ Interpretation Manual Background Staining (page 21)
	3b. Starch additives used in mounting sections to slides.	Avoid using starch additives for adhering sections to glass sides. Many additives are immunoreactive.	
	3c. Slides not thoroughly rinsed.	Ensure that the Autostainer is properly primed prior to running. Check to make sure that adequate buffer is provided for entire run. Use fresh solutions of buffers and washes.	HercepTest™ Interpretation Manual Background Staining (page 21)
	3d. Sections dried during staining procedure.	Verify that the appropriate volume of reagent is applied to slides. Make sure the Autostainer is run with the hood in the closed position and is not exposed to excessive heat or drafts.	HercepTest™ Interpretation Manual Background Staining (page 21)
	3e. Sections dried while loading the Autostainer.	Ensure sections remain wet with buffer while loading and prior to initiating run.	HercepTest™ Interpretation Manual Background Staining (page 21)
	3f. Inappropriate fixation method used.	Ensure that approved fixative was used. Alternative fixative may cause excessive background staining.	HercepTest™ Interpretation Manual Background Staining (page 21)
	3g. Non-specific binding of reagents to tissue.	Check fixation method of the specimen and presence of necrosis.	HercepTest™ Interpretation Manual Background Staining (page 21)
	3h. Excessive heating of tissue. Refer to 2d.	Use corrective procedure for drying tissue sections.	
4. Tissue detaches from slides.	4a. Use of incorrect slides.	Use silanized slides, such as Dako Silanized Slides, Code S3003, SuperFrost Plus or poly-L-lysine coated slides.	
5. Excessively strong specific staining.	5a. Inappropriate fixation method used.	Ensure that only approved fixatives and fixation methods are used.	
	5b. Use of improper heat source for epitope retrieval, e.g. steamer, microwave oven or autoclave.	Ensure that only an approved procedure for target retrieval is applied. Refer to the Instructions for Use.	
	5c. Reagent incubation times too long.	Review Staining Procedure instructions.	
	5d. Inappropriate wash solution used.	Use only the Wash Buffer that is recommended for the kit.	

Troubleshooting Guideline for HercepTest™

Problem	Probable Cause	Suggested Action	Reference
6. Weak staining of the 1+ Control Slide Cell Line.	6a. Incorrect epitope retrieval protocol followed.	Immerse the slides in the pre-heated Epitope Retrieval Solution. Bring temperature of the Epitope Retrieval Solution back to 95-99 °C and pre-treat for a full 40 minutes.	
	6b. Lack of reaction with Substrate-Chromogen Solution (DAB).	Ensure that the full 10 minute incubation time is used. Ensure that only one drop of DAB Chromogen was added to 1 mL of DAB Buffered Substrate.	
	6c. Degradation of Control Slide.	Check kit expiration date and kit storage conditions on outside of package.	
7. Other artifacts, miscellaneous.	7a. Heterogeneous Staining		HercepTest™ Interpretation Manual Heterogeneous Staining (page 19)
	7b. Cytoplasmic Staining		HercepTest™ Interpretation Manual Cytoplasmic Staining (page 23)
	7c. Edge Artifacts		HercepTest™ Interpretation Manual Edge Artifacts (page 24)
	7d. Retraction Artifacts		HercepTest™ Interpretation Manual Retraction Artifacts (page 25)
	7e. Thermal Artifacts		HercepTest™ Interpretation Manual Thermal Artifacts (page 26)
	7f. Crush Artifacts		HercepTest™ Interpretation Manual Crush Artifacts (page 27)
	7g. Decalcification Artifacts		HercepTest™ Interpretation Manual Decalcification Artifacts (page 27)

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Acknowledgements

Dako would like to thank Dr. Froilan Espinoza at Quest Diagnostics and Dr. Jim Thompson at Impath Laboratories for their generosity in contributing to this project. Impath Laboratories and Quest Diagnostics process a large volume of HercepTest™ slides every month. Dr. Espinoza and Dr. Thompson contributed by offering their expertise and providing many of the images throughout this manual.

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