HercepTest™ for the Dako Autostainer

Code K5207

1st edition

For immunocytochemical staining.

The kit is for 50 tests (100 slides).

Contents

Intended Use

For in vitro diagnostic use.

HercepTest™ is a semi-quantitative immunocytochemical 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 metastatic gastric or gastroesophageal junction adenocarcinoma. HercepTest™ is indicated as an aid in the assessment of patients for whom Herceptin® (trastuzumab) treatment is being considered (see Herceptin® package insert).

NOTE for breast cancer only: All of the patients in the Herceptin® clinical trials were selected using an investigational immunocytochemical clinical trial assay (CTA). None of the patients in those trials were selected using the HercepTest™. The HercepTest™ was compared to the CTA on an independent set of samples and found to provide acceptably concordant results. The actual correlation of the HercepTest™ to Herceptin® clinical outcome has not been established.

NOTE for gastric cancer only: All of the patients in the phase III BO18255 (ToGA) study sponsored by Hoffmann-La Roche were selected using Dako HercepTest™ (IHC) and Dako *HER2* FISH pharmDx™ Kit (FISH). However, enrollment in the BO18255 study was limited to patients whose tumors were HER2 protein overexpressing (IHC 3+) or gene amplified (FISH+; HER2/CEN-17 ratio ≥ 2.0). No patients were enrolled whose tumors were not gene amplified but HER2 protein weakly to strongly overexpressing [FISH(-)/IHC 2+], therefore it is unclear if patients whose tumors are not gene amplified but HER2 protein overexpressing [i.e., FISH(-), IHC 2+ or 3+] will benefit from Herceptin® treatment. The study also demonstrated that gene amplification and protein overexpression (IHC) are not as correlated as with breast cancer, therefore a single method should not be used to determine HER2 status.

For the present kit, Code K5207, reagent volumes have been adjusted especially for use with the Autostainer.

Gastric or gastroesophageal junction adenocarcinoma is also referred to as gastric cancer in this document.

For breast cancer application, please refer to pages 3-22.

For gastric cancer application, please refer to pages 23-44.

Important: Please note for breast cancer tissue and gastric cancer tissue differences especially in the Interpretation of Staining Sections.

Summary and Explanation - Breast

Background

The human *HER2* gene (also known as *ERBB2* or *NEU*) encodes a protein often referred to as HER2 protein or $p185^{HER2}$. The HER2 protein is a membrane receptor tyrosine kinase with homology to the epidermal growth factor receptor (EGFR or HER1) (1-8). The HER2 protein is a normal component expressed by a variety of epithelial cell types (8).

In a fraction of patients with breast cancer, the HER2 protein is overexpressed as part of the process of malignant transformation and tumor progression (9). Overexpression of the HER2 protein on the surface of breast cancer cells suggested that it could be a target for an antibody therapeutic. Herceptin® (trastuzumab) is a humanized monoclonal antibody (10) that binds with high affinity to the HER2 protein and has been shown to inhibit the proliferation of human tumor cells that overexpress HER2 protein in vitro and in vivo (11-13).

Characteristics

HercepTest™ was developed to provide an alternative to the investigational CTA used in the Herceptin® clinical studies. The performance of HercepTest™ for determination of HER2 protein overexpression was evaluated in an independent study comparing the results of the HercepTest™ to the CTA on 548 breast tumor specimens, none of which were obtained from patients in the Herceptin® clinical studies. The results indicated a 79% concordance between the results from the two assays on these tissue specimens.

The concordance data also indicates that a 3+ reading with HercepTest™ was highly likely to correspond with a positive reading on the CTA which would have met the entry criteria for the trial (2+ or 3+). A finding of 2+ on HercepTest™ did not correlate as well with the CTA results. Approximately 42% (53/126) of HercepTest™ 2+ results were negative by CTA (0 - 1+) which would not have allowed entry into the Herceptin® clinical trials.

HercepTest™ is interpreted as negative for HER2 protein overexpression (0 and 1+ staining intensity), weakly positive (2+ staining intensity), and strongly positive (3+ staining intensity). HercepTest™ is not intended to provide prognostic information to the patient and physician and has not been validated for that purpose.

Principle of Procedure – Breast

The HercepTest™ contains reagents required to complete a two-step immuno-cytochemical staining procedure for routinely processed, paraffin-embedded specimens. Following incubation with the primary rabbit 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 immunoglobulin molecules and horseradish peroxidase molecules linked to a common dextran polymer backbone, thus eliminating the need for sequential application of link antibody and peroxidase-conjugated antibody. Cross-reaction of the Visualization Reagent with human immunoglobulins and fetal calf serum has been removed by solid-phase absorption. 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. Results are interpreted using a light microscope.
Control Slides containing three formalin-fixed, paraffin-embedded human breast cancer cell lines with staining intensity scores of 0, 1+, and 3+ are provided to validate staining runs. The staining intensity of these cell lines has been correlated to the number of receptors per cell.

HercepTest™, Code K5207, is applicable for automated staining using the Autostainer.

Reagents - Breast

Materials provided

The materials listed below are sufficient for 50 tests (50 slides incubated with Primary Antibody to HER2 Protein and 50 slides incubated with the corresponding Negative Control Reagent). The number of tests is based on the use of 200 µL per slide of vials Nos. 1, 2, 3 and 4, and of the Substrate-Chromogen Solution (DAB). The kit provides materials sufficient for a maximum of 15 individual staining runs.

Vial No.	Quantity	Description
1	2 x 11 mL	PEROXIDASE-BLOCKING REAGENT Peroxidase-Blocking Reagent: 3% hydrogen peroxide containing 15 mmol/L sodium azide (NaN_3).
2	1 x 12 mL	RABBIT ANTI-HUMAN HER2 PROTEIN Rabbit Anti-Human HER2 Protein: Ready-to-use affinity-isolated antibody. Supplied in 0.05 mol/L Tris/HCl, 0.1 mol/L NaCl, 15 mmol/L NaN_3, pH 7.2, containing stabilizing protein. Immunogen: Synthetic C-terminal fragment (intracytoplasmic part) of the HER2 protein coupled to keyhole limpet hemocyanin. Specificity: HER2 protein. Purification method: The antibody is affinity isolated by using an immobilized HER2 protein peptide.
3	2 x 11 mL	VISUALIZATION REAGENT Visualization Reagent: Dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-rabbit immunoglobulins. Supplied in Tris/HCl buffer containing stabilizing protein and an antimicrobial agent.
4	1 x 12 mL	NEGATIVE CONTROL REAGENT Negative Control Reagent: Immunoglobulin fraction of normal rabbit serum at an equivalent protein concentration as the antibody to HER2 protein. Supplied in 0.05 mol/L Tris/HCl, 0.1 mol/L NaCl, 15 mmol/L NaN_3, pH 7.2, containing stabilizing protein.
5	15 x 11 mL	DAB BUFFERED SUBSTRATE DAB Buffered Substrate: Substrate buffer solution, pH 7.5, containing <0.1% hydrogen peroxide, stabilizers, enhancers, and an antimicrobial agent.
6	3 x 3 mL	DAB CHROMOGEN DAB Chromogen: 5% 3,3'-diaminobenzidine tetrahydrochloride chromogen solution.
7	1 x 1 L	EPITOPE RETRIEVAL SOLUTION (X 10) Epitope Retrieval Solution (x 10): 0.1 mol/L citrate buffer with an antimicrobial agent.
8	2 x 1 L	WASH BUFFER (10x) Wash Buffer (10x): Tris/HCl buffer with a detergent and an antimicrobial agent.
	3 x 5 slides	CONTROL SLIDES Control Slides: Each slide contains sections of three formalin-

fixed, paraffin-embedded breast carcinoma cell lines representing different levels of HER2 protein expression: MDA-231 (0), MDA-175 (1+), and SK-BR-3 (3+). The Control Slides have been heat treated for better adherence of sections to glass slides. Any additional heat treatment of Control Slides performed to improve the adherence of sections to glass slides may compromise staining results.

NOTE: All reagents, including Epitope Retrieval Solution and Wash Buffer, are formulated specifically for use with this test. For the test to perform as specified, no substitutions should be made except for the Wash Buffer, where Dako Code S3006 may be used.

Materials required but not provided

Ammonium hydroxide, 15 mol/L diluted to 37 mmol/L

Counterstain: Hematoxylin, such as water-based Mayer's Hematoxylin, Dako Code S3301 (see INSTRUCTIONS FOR USE, A.4)

Coverslips

Distilled or deionized water (Washing Water)

Drying oven, capable of maintaining 60 °C or less

Ethanol, absolute and 95%

Light microscope (4–40x objective magnification)

Mounting medium, such as Dako Faramount, Code S3025, or Glycergel™, Code C0563

Positive and Negative Tissues to use as process controls (see Quality Control Section)

Slides, SuperFrost Plus, poly-L-lysine-coated slides, or Dako Silanized Slides, Code S3003, (see Specimen Preparation)

Staining jars or baths

Timer (capable of 2–40 minute intervals)

Water bath with lid (capable of maintaining Epitope Retrieval Solution at 95–99 °C)

Xylene, toluene, or xylene substitutes

K5207 has been tailored for use with the Autostainer Immunostaining System, Code S3400. Please refer to the Autostainer User Guide for necessary Autostainer Components.

Storage – Breast

Store at 2–8 °C.

Do not use the kit after the expiration date stamped on the outside package. If reagents are stored under any conditions other than those specified in this package insert, they must be validated by the user (14a, 14b). Note that Control Slides must also be stored at 2–8 °C.

There are no obvious signs to indicate instability of this product. Therefore, Positive and Negative Controls should be run simultaneously with patient specimens. If unexpected staining is observed, which cannot be explained by variations in laboratory procedures, and a problem with HercepTest™ is suspected, immediately contact Dako's Technical Services.

Specimen Preparation - Breast

Specimens from biopsy must be handled to preserve the tissue for immunocytochemical staining. Standard methods of tissue processing should be used for all specimens (15).

Paraffin-embedded sections

Tissues preserved in neutral buffered formalin or Bouin's fixative for routine processing and paraffin embedding are suitable for use. For example, specimens from the biopsy should be blocked into a thickness of 3 or 4 mm and fixed for 18–24 hours in neutral buffered formalin. The tissues are then 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) (15, 16). In the USA, the Clinical Laboratory Improvement Act of 1988 requires in 42 CFR 493.1259(b) that "The laboratory must retain stained slides at least ten years from the date of examination and retain specimen blocks at least two years from the date of examination" (16).

Tissue specimens should be cut into sections of 4–5 µm, mounted onto slides and 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. **CAUTION:** Excessive heating for more than one hour at ≥60 °C may cause a significant decrease or loss of the specific membrane-associated HER2 immunoreactivity (17).

To preserve antigenicity, tissue sections mounted onto slides (SuperFrost Plus, poly-L-lysine or silanized slides) should be stained within 4-6 weeks of sectioning when stored at room temperature (20–25 °C) (18). The slides required for HER2 protein evaluation and verification of tumor presence should be prepared at the same time. A minimum of 5 slides is recommended, 1 slide for tumor presence, 2 slides for HER2 protein evaluation (1 for incubation with vial No. 2 and 1 for incubation with vial No. 4), and 2 slides for back-up.

The use of HercepTestTM on decalcified tissues has not been validated and is not recommended.

Consult Dako's "Education Guide: Immunohistochemical Staining Methods" (19) and references 15 and 16 for further details on specimen preparation.

Treatment of tissues prior to staining

A specific epitope retrieval method in 10 mmol/L citrate buffer, must be used for optimal assay performance. The Epitope Retrieval Solution is supplied in the HercepTest™ kit. This method involves heating of tissue sections mounted on slides that are immersed in 10 mmol/L citrate buffer (20) in a calibrated water bath capable of maintaining the Epitope Retrieval Solution at the required temperature (95–99 °C). Laboratories located at higher elevations should determine the best method of maintaining the required water bath temperature. The epitope retrieval must be performed in a water bath. Other methods of heating have been tested and do not give reproducible results. Immediately after epitope retrieval, commence the staining procedure. Deviation from the described procedure may affect results.

Precautions - Breast

1. For in vitro diagnostic use.
2. For professional users.
3. Vial 1, Peroxidase-Blocking Reagent, contains 3% hydrogen peroxide. Safety data sheet available for professional users on request.
4. Vial 6, DAB Chromogen, contains 1-5% 3,3'-diaminobenzidine tetrahydrochloride (biphenyl-3,3',4,4'-tetrayltetraammonium tetrachloride) and is labeled:
   Harmful.
   R40 Limited evidence of a carcinogenic effect.
   R43 May cause sensitization by skin contact.

R68 Possible risk of irreversible effects.
S35 This material and its container must be disposed of in a safe way.
S36/37 Wear suitable protective clothing and gloves.
As a general rule, persons under 18 years of age are not allowed to work with this product. Users must be carefully instructed in the proper work procedure, the dangerous properties of the product and the necessary safety instructions (per European Union Directive 94/33/EC). Please refer to the Material Safety Data Sheet (MSDS) for additional information.

USA: 3,3'-diaminobenzidine (DAB) may be harmful by inhalation, in contact with skin and if swallowed. Material is irritating to eyes and skin. If skin contact should occur, rinse affected areas with soap and water.

NOTE: Although diaminobenzidine is structurally related to benzidine, there is no evidence for the carcinogenicity of diaminobenzidine. Consult Federal, State, or local regulations for disposal.

5. Vial 8, Wash Buffer, contains 5-bromo-5-nitro-1,3-dioxane that may produce an allergic reaction. Safety data sheet available for professional users on request.
6. This product contains sodium azide (NaN_3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, sodium azide may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing (21, 22).
7. Vial 2, 3 and 4 contain material of animal origin. As with any product derived from biological sources, proper handling procedures should be used.
8. Control slides and specimens, before and after fixation, and all materials exposed to them, should be handled as if capable of transmitting infection, and disposed of with proper precautions (23). Never pipette reagents by mouth and avoid contacting the skin and mucous membranes with reagents and specimens. If reagents come in contact with sensitive areas, wash with copious amounts of water.
9. Minimize microbial contamination of reagents to avoid non-specific staining.
10. Incubation times, temperatures, or methods other than those specified may give erroneous results. Excess drying at ≥60 °C for more than one hour may cause a significant decrease or loss of the specific membrane-associated HER2 immunoreactivity (17).
11. Reagents have been optimally diluted. Further dilution may result in loss of antigen staining.
12. All reagents, including Epitope Retrieval Solution and Wash Buffer, are formulated specifically for use with this test. In order for the test to perform as specified, no substitutions should be made except for the Wash Buffer, where Code S3006 may be used.
13. The Visualization Reagent and DAB Chromogen may be affected adversely if exposed to excessive light levels. Do not store system components or perform staining in strong light, such as direct sunlight.
14. Wear appropriate personal protective equipment to avoid contact with eyes and skin. Refer to the Material Safety Data Sheet (MSDS) for additional information.

INSTRUCTIONS FOR USE - Breast

A. Reagent Preparation

It is convenient to prepare the following reagents prior to staining:

A.1 Epitope Retrieval Solution

Dilute a sufficient quantity of Vial 7 (Epitope Retrieval Solution x 10) 1:10 using distilled or deionized water for the staining procedure that is planned. Unused diluted solution may be stored at 2–8 °C for one month. Discard diluted solution if cloudy in appearance.

A.2 Wash Buffer

Dilute a sufficient quantity of Vial 8 (Wash Buffer x 10) 1:10 using distilled or deionized water for the wash steps. Unused diluted buffer may be stored at 2–8 °C for one month. Discard buffer if cloudy in appearance.

The Autostainer is programmed to rinse tissue sections after the Peroxidase-Blocking Reagent and Substrate-Chromogen Solution. Note that for these rinse steps either distilled (or deionized) water or Wash Buffer may be used. All other rinse steps require the use of Wash Buffer.

A.3 Substrate-Chromogen Solution (DAB)

Prepare Substrate-Chromogen Solution by adding 11 drops (25–30 □L per drop) of DAB Chromogen from Vial 6 to one Vial 5 containing DAB Buffered Substrate (11 mL) and mix. Prepared Substrate-Chromogen Solution (DAB) is stable for approximately 5 days when stored at 2–8 °C. This solution should be mixed thoroughly prior to use. Any precipitate developing in the solution does not affect staining quality.

NOTE: The color of the DAB Chromogen in Vial 6 may vary from clear to light lavender-brown. This will not affect the performance of this product. Please dilute according to the guidelines in this package insert. Addition of excess DAB Chromogen to the DAB Buffered Substrate will result in deterioration of the positive signal.

A.4 Counterstain

The colored end-product of the DAB staining reaction is alcohol and water insoluble. Use a hematoxylin counterstain and adjust the hematoxylin staining intensity to a similar level shown in Dako's "HercepTest™ Interpretation Manual – Breast Cancer". Hematoxylin, either alcohol or water-based, such as Dako Mayer's Hematoxylin, Code S3301, may be used.
Follow hematoxylin counterstaining with a thorough rinse in distilled or deionized water, then immerse tissue slides into a bath of 37 mmol/L ammonia water (see Section B.2, Step 3). Ammonia water (37 mmol/L) is prepared by mixing 2.5 mL of 15 mol/L (concentrated) ammonium hydroxide with 1 liter of distilled or deionized water. Unused 37 mmol/L ammonia water may be stored at room temperature (20–25 °C) in a tightly capped bottle for up to 12 months.

A.5 Mounting Medium

Non-aqueous, permanent mounting medium is recommended. However, aqueous mounting is also acceptable. Dako Faramount Aqueous Mounting Medium, Ready-to-Use, Code S3025, or Glycergel™ Mounting Medium, Code C0563, is recommended for aqueous mounting. Liquefy Glycergel™ by warming to approximately 40 (± 5) °C prior to use.

B. Staining Procedure Performed on the Autostainer

B.1 Procedural notes

The user should read these instructions carefully and become familiar with all components and instrumentation prior to use (see Precautions).

All reagents should be equilibrated to room temperature (20–25 °C) prior to immunostaining. Likewise, all incubations should be performed at room temperature.

Do not allow tissue sections to dry while loading slides on the Autostainer and during the staining procedure. Dried tissue sections may display increased non-specific staining.

If the staining procedure is interrupted, slides may be kept in a buffer bath following incubation of the primary antibody for up to one hour at room temperature (20–25 °C) without affecting the staining performance.

Deparaffinization and rehydration: Prior to staining, tissue slides must be deparaffinized to remove embedding medium and rehydrated. Avoid incomplete removal of paraffin. Residual embedding medium will result in increased non-specific staining. This step should be performed at room temperature (20–25 °C).

1. Place slides in a xylene bath and incubate for 5 (±1) minutes. Change baths and repeat once.
2. Tap off excess liquid and place slides in absolute ethanol for 3 (±1) minutes. Change baths and repeat once.
3. Tap off excess liquid and place slides in 95% ethanol for 3 (±1) minutes. Change baths and repeat once.
4. Tap off excess liquid and place slides in distilled or deionized water for a minimum of 30 seconds. Commence staining procedure as outlined in Section B.2, Step 1, Epitope retrieval.

Xylene and alcohol solutions should be changed after 40 slides. Toluene or xylene substitutes, such as Histoclear, may be used in place of xylene.

NOTE: The reagents and instructions supplied in this kit have been designed for optimal performance. Further dilution of the reagents or alteration of incubation temperatures may give erroneous or discordant results. Differences in tissue processing and technical procedures in the user's laboratory may invalidate the assay results for use in selecting patients for Herceptin® therapy.

B.2 Staining protocol

Performed at room temperature, 20–25 °C.

Step 1: Epitope retrieval

Fill staining jars, e.g. Coplin jars, with the diluted Epitope Retrieval Solution (see INSTRUCTIONS FOR USE, Section A.1). Place staining jars containing Epitope Retrieval Solution in water bath. Heat water bath and the Epitope Retrieval Solution to 95–99 °C. Cover jars with lids to stabilize the temperature and avoid evaporation.

Immerse the room temperature deparaffinized sections in the preheated Epitope Retrieval Solution in the staining jars. **BRING TEMPERATURE OF THE WATER BATH AND THE EPITOPE RETRIEVAL SOLUTION BACK TO 95–99 °C**. Incubate for 40 (±1) minutes at 95–99 °C.

Remove the entire jar with slides from the water bath. Allow the slides to cool in the Epitope Retrieval Solution for 20 (±1) minutes at room temperature.

Decant the Epitope Retrieval Solution and rinse sections in the Wash Buffer (see INSTRUCTIONS FOR USE, Section A.2).

For optimal performance, soak sections in Wash Buffer for 5–20 minutes after epitope retrieval and prior to staining.

NOTE: The Epitope Retrieval Solution is designed for single use application only. Do not re-use.

Step 2: Autostainer procedure

1. Use the Autostainer-generated map on the HercepTest™ autoprogram for required program times and reagent volumes (see point 4 below for specific volumes).
2. Place the Autostainer reagent vials in the Autostainer reagent rack according to the computer-generated reagent map.
3. Load the slides onto the Autostainer according to the computer- generated slide map.
4. Set Program and begin the HercepTest™ program. The following is an outline of the program run:
   - Rinse
   - 200 µL Peroxidase-Blocking Reagent - 5 minutes
   - Rinse
   - 200 µL Primary Anti-HER2 Protein (or Negative Control Reagent) - 30 minutes
   - Rinse
   - 200 µL Visualization Reagent - 30 minutes
   - Rinse
   - Rinse
   - Switch
   - 200 µL Substrate-Chromogen Solution (DAB) - 10 minutes

   Rinse slides in deionized water after the substrate-chromogen step

NOTE: Autostainer Hardware version 01 rinses slides in buffer. Therefore, the slides must be rinsed with deionized water after they have been removed from the Autostainer.

Step 3: Counterstain (instructions are for hematoxylin)

Remove slides from the Autostainer and counterstain in hematoxylin as described below.

Immerse slides in a bath of hematoxylin. Incubate for 2–5 minutes, depending on the strength of hematoxylin used.

Rinse *gently* in a distilled or deionized water bath. Ensure all residual hematoxylin has been cleared.

Optional: Dip slides 10 times into a bath of 37 mmol/L ammonia water (see Section A.4).

Rinse slides *gently* in a bath of distilled or deionized water for 2–5 minutes.

NOTE: Depending on the incubation length and potency of the hematoxylin used, counterstaining will result in a pale to dark blue coloration of the cell nuclei. Excessive or incomplete counterstaining may compromise proper interpretation of results.

Step 4: Mounting

Non-aqueous, permanent mounting medium is recommended. Otherwise, aqueous mounting medium is also acceptable. Specimens may be mounted and coverslipped with a water-based mounting medium such as Dako Faramount, Code S3025, or Glycergel™, Code C0563.

NOTE: Slides may be read when convenient. However, some fading may occur if slides are coverslipped with an aqueous mounting medium and exposed to strong light over a period of one week. To minimize fading, store slides in the dark at room temperature (20–25 °C).

Quality Control - Breast

Differences in tissue fixation, processing and embedding in the user's laboratory may produce significant variability in results, necessitating regular performance of in-house controls in addition to the Control Slides supplied by Dako. In the USA, consult the quality control guidelines of the College of American Pathologists (CAP) Certification Program for Immunohistochemistry, see also CLSI (formerly NCCLS) Quality Assurance for Immunocytochemistry, Approved Guideline (24), and reference 25 for additional information.

Table 1. The purpose of daily quality control.

Tissue: Fixed and Processed Like Patient Sample	Specific Antibody and Secondary Antibody	Non-Specific Antibody* or Buffer Plus Same Secondary Antibody as Used with Specific Antibody
Positive Control: Tissue or cells containing target antigen to be detected (could be located in patient tissue). The ideal control is weakly positive staining tissue as this is most sensitive to antibody or antigen degradation	Controls all steps of the analysis. Validates reagent and procedures used for HER2 protein staining	Detection of non-specific background staining
Negative Control: Tissues or cells expected to be negative (could be located in patient tissue or positive control tissue)	Detection of unintended antibody cross-reactivity to cells/cellular components	Detection of non-specific background staining
Patient tissue	Detection of specific staining	Detection of non-specific background staining
Control Slide supplied by Dako	Controls staining procedure only	

* Serum from same species as the specific antibody, but not directed against the same target antigen. To detect non-specific antibody binding, e.g. binding of Fc portion of antibody by the tissue.

Control Slide (provided): Each of the supplied Control Slides contains three pelleted, formalin-fixed, paraffin-embedded human breast cancer cell lines with staining intensity scores of 0, 1+ and 3+. One slide should be stained in each staining run. The evaluation of the Control Slide cell lines supplied by Dako indicates the validity of the staining run.

Positive Control Tissue: Controls should be fresh autopsy/biopsy/surgical specimens fixed, processed and embedded as soon as possible in the same manner as the patient sample(s). Positive tissue controls are indicative of correctly prepared tissues and proper staining techniques. One Positive Control Tissue for each set of test conditions should be included in each staining run.

The Positive Control Tissues should give weak positive staining so they can detect subtle changes in the primary antibody sensitivity. The Control Slides supplied with this kit or specimens processed differently from the patient sample(s) validate reagent performance only and do not verify tissue preparation. Use previously determined HER2 protein 2+ overexpressing invasive (infiltrating) human breast carcinoma tissue for the ideal Positive Control Tissue.

NOTE: Known positive control tissue should only be utilized for monitoring the correct performance of processed tissues and test reagents, NOT as an aid in formulating a specific diagnosis of patient samples. If the Positive Control Tissue fails to demonstrate appropriate positive staining, results with the patient specimens should be considered invalid.

Negative Control Tissue: Use a negative control tissue (known to be HER2 protein negative) fixed, processed and embedded in a manner identical to the patient sample(s) with each staining run to verify the specificity of the primary antibody and to provide an indication of

specific background staining. Colon, liver or thyroid are appropriate for negative control tissue. The variety of different cell types present in most tissue sections offers internal negative control sites (this should be verified by the user). Normal breast ducts can serve as internal negative controls.

If specific staining occurs in the Negative Control Tissue or in the internal negative control tissue, results with the patient specimens should be considered invalid and the test re-run.

Non-Specific Negative Control Reagent: Use the supplied Negative Control Reagent in place of the primary antibody with a section of each patient specimen to evaluate non-specific staining and allow better interpretation of specific staining at the antigen site. The incubation period for the Negative Control Reagent should correspond to that of the primary antibody.

Assay verification: Prior to initial use of an antibody or staining system in a diagnostic procedure, the user should verify the antibody's specificity by testing it on a series of in-house tissues with known immunocytochemical performance characteristics representing known positive and negative tissues. Refer to the quality control procedures previously outlined in this section of the product insert and to the quality control requirements of the CAP Certification Program for Immunohistochemistry and/or CLSI (formerly NCCLS) Quality Assurance for Immunocytochemistry, Approved Guideline (24). These quality control procedures should be repeated for each new antibody lot, or whenever there is a change in assay parameters. Breast carcinomas with known HER2 protein staining intensities from 0 - 3+ and negative tissues, e.g. colon, liver or thyroid are suitable for assay verification.

Interpretation of Staining - Breast

For the determination of HER2 protein overexpression, only the *membrane* staining intensity and pattern should be evaluated using the scale presented in Table 2. Slide evaluation should be performed by a pathologist using a light microscope. For evaluation of the immunocytochemical staining and scoring, an objective of 10x magnification is appropriate. The use of a 20–40x objective magnification is useful in confirmation of the score. Cytoplasmic staining should be considered non-specific staining and is not to be included in the assessment of membrane staining intensity (8). To aid in the differentiation of 0, 1+, 2+ and 3+ staining, refer to Dako's "HercepTest™ Interpretation Manual – Breast Cancer" for representative pictures of the staining intensities.

Only specimens from 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.

Table 2. Cell membrane staining intensity criteria.

Staining pattern	**Score** (Report to treating physician)	**HER2 Protein Overexpression Assessment** (Report to treating physician)
No staining is observed or membrane staining is observed in less than 10% of the tumor cells	0	Negative
A faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane	1+	Negative
A weak to moderate complete membrane staining is observed in more than 10% of the tumor cells	2+	Weakly positive
A strong complete membrane staining is observed in more than 10% of the tumor cells	3+	Strongly positive

HercepTest™ is interpreted as negative for HER2 protein overexpression (0 and 1+ staining intensity), weakly positive (2+ staining intensity), and strongly positive (3+ staining intensity). HercepTest™ is not intended to provide prognostic information to the patient and physician and has not been validated for that purpose.

For each staining run, slides should be examined in the order presented in Table 3 to determine the validity of the staining run and enable semi-quantitative assessment of the staining intensity of the sample tissue.

Table 3. Order of slide evaluation.

Slide Reading Order	Rationale
1. Control Slide containing the three cell lines	Presence of 3+ brown cell membrane staining (rimming) in the 3+ Control Cell Line SK-BR-3, partial brown rimming in the 1+ Control Cell Line MDA-175, and no staining in the 0 Control Cell Line MDA-231 indicates a valid assay Punctate and discontinuous membrane staining is present in a small to moderate number of the cells in the weakly positive 1+ Control Cell Line MDA-175. Also dot-like immunostaining of the Golgi region of the cytoplasm can be observed in this cell line Presence of brown staining in the 0 Control Cell Line MDA-231 (negative for HER2 protein staining) indicates that there was non-specific staining during the assay. The assay results may be invalid due to overstaining
2. Positive Control Tissue Slide	Presence of brown membrane staining should be observed. Staining of the cytoplasm and negative tissues should not be more than 1+
3. Negative Control Tissue Slide	The **ABSENCE** of specific staining in the Negative Control Tissue Slide confirms the lack of kit cross-reactivity to cells/cellular components. If specific membrane staining occurs in the Negative Control Tissue Slide, results with the patient specimen should be considered invalid
4. Patient tissue slide stained using the Negative Control Reagent	Absence of specific membrane staining verifies the specific labeling of the target antigen by the primary antibody Other tan or brown staining occurring in the cytoplasm of the specimen treated with the Negative Control Reagent, such as in connective tissue, leukocytes, erythrocytes, or necrotic tissue, should be considered non-specific background staining and should be reported under the comments section of the data spreadsheet
5. Patient tissue slide stained using the primary antibody	When HER2 protein overexpression is detected in the specimen, it will appear as brown rimming localized on the cell membrane of tumor cells treated with the primary antibody

1. Control Slide (provided): The Control Slide stained with HercepTest™ should be examined first to ascertain that all reagents are functioning properly. The presence of a brown (3,3'-diaminobenzidine, DAB) reaction product at the cell membrane is indicative of positive reactivity.

Presence of circumferential brown cell membrane staining (rimming) in the 3+ Control Cell Line SK-BR-3, partial brown rimming in the 1+ Control Cell Line MDA-175, and no staining in the 0 Control Cell Line MDA-231 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.

2. Positive Control Tissue: The Positive Control Tissue Slide should be examined next. This slide verifies that the fixation method and epitope retrieval process are effective. Use intact cells for interpretation of staining results because necrotic or degenerated cells often stain non-specifically (26). Staining should be observed in tumor tissue as brown, cell membrane staining. Brown staining of the cytoplasm and negative tissues within the specimen should not be more than corresponding to 1+ staining intensity score.

3. Negative Control Tissue: The Negative Control Tissue Slide should be examined after the Positive Control Tissue to verify the specificity of the labeling of the target antigen by the primary antibody. The absence of specific staining in the Negative Control Tissue confirms the lack of kit cross-reactivity to cells/cellular components. If specific staining occurs in the Negative Control Tissue, results with the patient specimen should be considered invalid. Alternatively, negative portions of the Positive Control Tissue may serve as the Negative Control Tissue, but this should be verified by the user. Note that a weak reaction (0 - 1+

staining intensity) can be observed in most normal epithelial tissue. Possible negative control tissues include: colon, liver and thyroid.

Non-specific staining, if present, will be of a diffuse appearance. Sporadic staining of connective tissue may also be observed in sections from excessively formalin-fixed tissues.

4 + 5. Patient Tissue: Examine patient specimens stained with HercepTest™ last. Positive staining intensity should be assessed within the context of any non-specific background staining of the Negative Control Reagent. As with any immunocytochemical test, a negative result means that the antigen was not detected, not that the antigen was absent in the cells/tissue assayed. Refer to Summary and Explanation, Limitations, and Performance Characteristics for specific information regarding HercepTest™ immunoreactivity.

Additional Recommendations for Interpretation of HercepTest™ Staining
Most metastatic breast carcinomas tested for HER2 protein overexpression are given a score of 0 or 3+. While the majority of these cases are clear-cut, a small percentage of the remaining 1+ and 2+ samples may be more difficult to interpret. Use the following guidelines for interpretation of HercepTest™ staining in your laboratory.

- Evaluate the Control Cell Lines to validate the assay performance.
- Evaluate the Positive and Negative Control Slides.
- A hematoxylin and eosin (H&E) staining 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 is required from the pathologist to verify the presence of the tumor). The HercepTest™ should be performed on a paired section (serial section) from the same paraffin block of the specimen.
- Evaluate the sections stained for HER2 protein overexpression at low power first. The majority of positive cases will be obvious at low power magnification.
- Well-preserved and well-stained areas of the specimen should be used to make a determination of the percent positive tumor cells.
- In general, the score of cases should be obvious at low magnification. If determination between 1+/2+ borderline cases is difficult at low magnification, the score is usually 1+.
- To verify membrane staining, use 20–40x objective magnification.
- If a majority of tumor cells demonstrate complete membrane staining, the staining is either 2+ or 3+. Go to 20–40x objective magnification to confirm score.
- In a majority of the 3+ cases, at least 80% of the tumor cells are stained and the membrane staining is intense.
- If the specimen is near the cut off of 10% tumor cells positive, it is recommended that a minimum of 100 tumor cells be counted to determine the percentage of stained cells.
- If there is complete membrane staining at a weak to moderate intensity in greater than 10% of the tumor cells, the score of the specimen is 2+. This is usually accompanied by incomplete membrane staining of the majority of the remaining tumor cells.
- If less than 10% of the tumor cells have complete circumferential membrane staining, although other tumor cells may demonstrate an incomplete membrane staining, the score is 1+.
- If less than 10% of the tumor cells have complete or incomplete circumferential membrane staining, the score is 0.

Limitations - Breast

General limitations

1. Immunocytochemistry is a multi-step diagnostic process that requires specialized training in the selection of the appropriate reagents; tissue selection, fixation, and processing; preparation of the immunocytochemistry slide; and interpretation of the staining results.
2. Tissue staining is dependent on the handling and processing of the tissue prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, sectioning, or contamination with other tissues or fluids may produce artifacts, antibody trapping, or false-negative results. Inconsistent results may be due to variations in fixation and embedding methods, or to inherent irregularities within the tissue.
3. Excessive or incomplete counterstaining may compromise proper interpretation of results.
4. The clinical interpretation of any positive staining or its absence must be evaluated within the context of clinical presentation, morphology and other histopathological criteria. The clinical interpretation of any staining, or its absence, must be complemented by morphological studies and proper controls as well as other diagnostic tests. It is the responsibility of a qualified pathologist, who is familiar with the antibodies, reagents and methods used, to interpret the stained preparation. Staining must be performed in a certified, licensed laboratory under the supervision of a pathologist who is responsible for reviewing the stained slides and assuring the adequacy of positive and negative controls.
5. Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit non-specific staining with horseradish peroxidase (27).
6. Reagents may demonstrate unexpected reactions in previously untested tissue types. The possibility of unexpected reactions even in tested tissue types cannot be completely eliminated due to biological variability of antigen expression in neoplasms, or other pathological tissues (28). Contact Dako's Technical Services with documented unexpected reaction.
7. False-positive results may be seen due to non-immunological binding of proteins or substrate reaction products. They may also be caused by pseudoperoxidase activity (erythrocytes) and endogenous peroxidase activity (cytochrome C) (28).
8. The staining procedure should be performed at ambient temperature of 20–25 °C.

Product-specific limitations

1. The antigen present in the 1+ Control Cell Line MDA-175 is subject to degradation over time. Assess the Control Slide results in connection with the expiration date of the Control Slide. Negative staining of the MDA-175 cells may only indicate that the Control Slide has degraded. The Control Slides must be stored at 2–8 °C.
2. False-negative results could be caused by degradation of the antigen in the tissues over time. Specimens should be stained within 4-6 weeks of mounting of tissues on slides when stored at room temperature (20–25 °C) (29).
3. For optimal and reproducible results, the HER2 protein requires heat-induced epitope retrieval when tissues are routinely fixed (neutral buffered formalin or Bouin's fixative) and paraffin embedded. This pre-treatment needs to be completed at the beginning of the entire staining process. See the "Specimen Preparation Section, Treatment of tissues prior to staining" for instructions.
4. Heat-induced epitope retrieval of the HER2 protein should only be done using a calibrated water bath. Other methods of heating have been tested and do not give reproducible results.
5. Do not replace kit reagents with reagents carrying other lot numbers or with reagents from other manufacturers. The only exception is the Wash Buffer that may be replaced with Dako Wash Buffer, Code S3006.

6. False results could be obtained from evaluation of cytoplasmic staining. Consider only the intensity of cell membrane staining when interpreting results.
7. Stained Control Slides should be used only for validation of the staining run and should *not* be used as a guide to score the staining reaction in tissue sections.
8. Strong focal staining (3+), i.e. "hot spots," may occasionally be seen. This may be the result of uneven fixation and/or processing of tissue. Immunostaining of a second tissue block from the same specimen is recommended.
9. Use of HercepTest™ on specimens fixed in fixatives other than neutral buffered formalin or Bouin's fixative has not been validated.
10. Normal epithelium in breast tissue should stain between 0 and 1+. If higher than 1+ staining of the normal epithelium is observed, the test should be repeated. Note that normal tonsil and esophageal epithelia may stain up to 2+ intensity.

Performance Characteristics - Breast

Background

The clinical trial assay (CTA) used to identify eligible patients for the Herceptin® clinical studies was for investigational use and is no longer available. The HercepTest™ was developed to provide a comparable alternative to the CTA.

The safety and effectiveness of Herceptin® were evaluated in a randomized controlled clinical trial and a large, open-labelled trial (See Herceptin® package insert). All patients selected for the Herceptin® clinical trials demonstrated overexpression of HER2 protein by immunocytochemistry testing performed with the CTA at a central laboratory. Patients were eligible for Herceptin® treatment if their tumor had 2+ or 3+ levels of HER2 protein overexpression (based on a 0-3+ scale, where 3+ represented the highest level).

Subgroup analysis of the results from these studies suggests that patients whose tissues are strongly positive (3+) for HER2 protein overexpression may benefit more from Herceptin® than patients whose tissues are weakly positive (2+). The degree of HER2 protein overexpression is potentially an important predictor of the effect of Herceptin® treatment. Because none of the patients in the Herceptin® studies were selected using the HercepTest® the correlation between the degree of positivity and the likelihood of clinical benefit from Herceptin® treatment is unknown.

Comparison studies

Two studies were performed to characterize the HercepTest™.

1) Comparison to the Clinical Trial Assay (CTA).
2) Accuracy when compared with five additional assays.

Comparison to the Clinical Trial Assay (CTA)

The HercepTest™ was compared to the CTA used to identify eligible patients for Herceptin® therapy using 274 HER2 protein positive (2+ or 3+) and an equal number of HER2 protein negative breast cancer tissue specimens. Table 4 shows the results in a 2 x 2 diagram where 0 and 1+ were considered to be negative and 2+ and 3+ were positive.

Table 4. A 2 x 2 concordance of the HercepTest™ to the Clinical Trial Assay (number of specimens).

		Clinical Trial Assay		
		Positive	Negative	Total
HercepTest™	Positive	216	59	275
	Negative	58	215	273
	Total	274	274	548

Concordance: 79% (76–82%) 95% confidence interval.

The overall binary concordance of the HercepTest™ to the CTA was 79% (431/548), with a 2-sided 95% confidence interval of 76–82%. Twenty one per cent (21%) of the results were discordant between these two methods.

The HercepTest™ results are reported on a 0-3+ scale interpreted as negative for HER2 protein overexpression (0 and 1+ staining intensity), weakly positive (2+ staining intensity), and strongly positive (3+ staining intensity).

Table 5. A 3 x 3 concordance for HercepTest™ and Clinical Trial Assay.

		Clinical Trial Assay			
		3+	2+	0 - 1+	Total
HercepTest™	3+	107	36	6	149
	2+	16	57	53	126
	0 - 1+	8	50	215	273
	Total	131	143	274	548

This 3 x 3 presentation of the concordance study indicates that a 3+ reading on the HercepTest™ is highly likely to correspond with a positive result on the CTA, which would have met the entry criteria for the Herceptin® trial (2+ or 3+). A finding of 2+ on HercepTest™ did not correlate as well with the CTA results. Approximately 42% (53/126) of HercepTest™ 2+ results were negative by CTA (0 - 1+) which would not have allowed entry into the Herceptin® clinical trials.

Accuracy

HercepTest™ was also tested on 2 microscope slides containing paraffin-embedded tissue sections from 168 breast tumors. These tumors had been previously characterized by five different methods of determining *HER2* gene amplification and overexpression of HER2 protein, including in-house Southern blot, fluorescence in situ hybridization (FISH) for amplification of DNA, Northern blot RNA analysis, Western blot, and immunocytochemistry (ICC) on frozen tissues (29). The results are presented in Table 6.

Table 6. Comparison of HercepTest™ to combined results (OE) from gene amplification and HER2 protein overexpression tests.

		Reference OE Classification		
		+	-	Total
HercepTest™	+	43	0	43
	-	26	99	125
	Total	69	99	168

Positive agreement: 43/69 = 62%
Negative agreement: 99/99 = 100%

The results indicated an 85% (142/168) level of agreement (95% confidence interval of 78-89%) between the positivity (2+ and 3+) and negativity (0 and 1+) staining intensity by the

HercepTest™. None of the samples negative by the 5 different methods were positive by the HercepTest™, whereas the combined results for the 5 different methods showed a higher number of positive cases.

Reproducibility

Intra-run reproducibility: Intra-run reproducibility was tested in one laboratory with 5 specimens of different ICC intensity staining scores. Each specimen was run in triplicate in a masked randomized format. This protocol was used with automated staining. All specimens gave 100% reproducible results.

Inter-run reproducibility: Inter-run reproducibility was tested at three laboratories over 4 days with 5 specimens of different ICC intensity staining scores randomized and masked using automated methodology. Excellent reproducibility was seen for positive versus negative results (0 and 1+ versus 2+ and 3+) with the exception of two samples in one laboratory that varied between 1+ and 2+. There was 100% reproducibility for the 2+ and 3+ samples.

Inter-laboratory reproducibility: Inter-laboratory reproducibility was tested at three geographically separated laboratories with 40 identical randomized and masked specimens of various ICC staining intensity scores. Freshly cut slides were forwarded to each testing laboratory for automated staining and evaluation by a pathologist. Inter-laboratory percent agreement ranged from 83% to 90% for a dichotomous positive/negative determination where 0 and 1+ were negative and 2+ and 3+ were positive for HER2 protein overexpression. Compared to results obtained at the reference laboratory that had performed the CTA, 12.5% (15/120) comparative results were discrepant between negative (0 or 1+) and positive (2+ or 3+) determinations. An additional 10% (12/120) were discrepant between 2+ and 3+ scores.

Immunoreactivity

Table 7 summarizes HercepTest™ immunoreactivity with the recommended panel of normal tissues. All tissues were formalin fixed and paraffin embedded and stained with HercepTest™ according to the instructions in the package insert.

Table 7. Summary of HercepTest™ normal tissue reactivity.

Tissue Type (No. Tested)	Positive Tissue Element Staining and Staining Pattern
Adrenal (3)	None
Bone marrow (3)	None
Brain/Cerebellum (3)	None
Brain/Cerebrum (3)	None
Breast (3)	Mammary gland (1+ staining intensity)
Cervix uteri (3)	None
Colon (3)	Columnar epithelium, surface (1+ staining intensity)
Esophagus (3)	Squamous epithelia (1/3 tissues, 2+ staining intensity)
Heart (3)	None
Kidney (3)	Tubule (1+ staining intensity)
Liver (3)	None
Lung (3)	None
Mesothelial cells (3)	None
Ovary (3)	None
Pancreas (3)	Langerhans cells, cytoplasmic (3+ staining intensity)
Parathyroid (3)	None
Peripheral nerve (3)	None
Pituitary (3)	Endocrine cells, cytoplasmic (3+ staining intensity)
Prostate (3)	Prostate gland (2+ staining intensity)
Salivary gland (3)	None
Skeletal muscle (3)	None
Skin (3)	None
Small intestine (3)	Columnar epithelium, surface (1+ staining intensity)
Spleen (3)	None
Stomach (3)	Epithelium (1/3 tissues, 1+ staining intensity)
Testis (3)	None
Thymus (3)	None
Thyroid (3)	None
Tonsil (3)	Squamous epithelia (2+ staining intensity)
Uterus (3)	Endometrium (1/3 tissues, 1+ staining intensity)

Reported staining in all tissues was membrane, unless otherwise noted. All three specimens of each tissue type had the same staining intensity unless otherwise noted.

Troubleshooting - Breast

Refer to the Troubleshooting section in Dako's previously referenced Handbook (19) for remedial action, or contact Dako's Technical Service Department to report unusual staining.

Problem	Probable Cause	Suggested Action
1. No staining of slides	1a. Programming error. Reagents not used in proper order	1a. 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	1b. Check the Reagent Map to verify the proper location of reagent vials
	1c. Insufficient reagent in reagent vial	1c. Ensure that enough reagent is loaded into the reagent vials prior to commencing the run. Refer to Reagent Map for volumes required
	1d. Sodium azide in Wash Solution	1d. Use fresh preparation of Wash Buffer provided in the kit
	1e. Excessive heating of mounted tissue sections prior to deparaffinization and heat-induced antigen retrieval may lead to loss of visible HER2 immunoreactivity.	1e. 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 (17).
2. Weak staining of slides	2a. Inadequate epitope retrieval	2a. Verify that Epitope Retrieval Solution reaches 95–99 °C for a full 40 minutes and is allowed to cool for an additional 20 minutes
	2b. Inadequate reagent incubation times	2b. Review Staining Procedure instructions
	2c. Inappropriate fixation method used	2c. Ensure that patient tissue is not over-fixed or that an alternative fixative was not used
	2d. Excessive heating of mounted tissue sections prior to deparaffinization and heat-induced antigen retrieval may cause a significant decrease in visible HER2 immunoreactivity	2d. 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 (17).
3. Excessive background staining of slides	3a. Paraffin incompletely removed	3a. Use fresh clearing solutions and follow procedure as outlined in Section B.1
	3b. Starch additives used in mounting sections to slides	3b. Avoid using starch additives for adhering sections to glass slides.

		Many additives are immuno-reactive
	3c. Slides not thoroughly rinsed	3c. 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
	3d. Sections dried during staining procedure	3d. 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
	3e. Sections dried while loading the Autostainer	3e. Ensure sections remain wet with buffer while loading and prior to initiating run
	3f. Inappropriate fixation method used	3f. Ensure that approved fixative was used. Alternative fixative may cause excessive back-ground staining
	3g. Non-specific binding of reagents to tissue	3g. Check fixation method of the specimen and presence of necrosis
4. Tissue detaches from slides	4a. Use of incorrect slides	4a. 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	5a 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	5b. Ensure that only a water bath is used for the epitope retrieval step
	5c. Reagent incubation times too long	5c. Review Staining Procedure instructions
	5d. Inappropriate wash solution used	5d. Use only the Wash Buffer that is recommended for the kit
6. Weak staining of the 1+ Control Slide Cell Line	6a. Incorrect epitope retrieval protocol followed	6a. 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)	6b. 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	6c. Check kit expiration date and kit storage conditions on outside of package

NOTE: If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please call Dako's Technical Services for further assistance.

Additional information on staining techniques and specimen preparation can be found in Dako's previously referenced Handbook (19) (available from Dako), Atlas of Immunohistology (30) and Immunoperoxidase Techniques. A Practical Approach to Tumor Diagnosis (31)

Summary and Explanation - Gastric

Background

The human *HER2* gene (also known as *ERBB2* or *NEU*) encodes a protein often referred to as HER2 protein or $p185^{HER2}$. The HER2 protein is a membrane receptor tyrosine kinase with homology to the epidermal growth factor receptor (EGFR or HER1) (1-8). The HER2 protein is a normal component expressed by a variety of epithelial cell types (8).

Overexpression of the HER2 protein and amplification of the *HER2* gene in gastric cancer have been shown in a large number of studies (32). HER2 positivity can be detected in approximately 20% of the patients by either IHC or FISH (32). Preclinical in vitro and in vivo studies have demonstrated that trastuzumab (Herceptin®) is effective in different gastric cancer models thus leading to the initiation of several clinical studies (32-36).

All of the patients in the phase III BO18255 (ToGA) study sponsored by Hoffmann-La Roche were selected using Dako HercepTest™ (IHC) and Dako *HER2* FISH pharmDx™ Kit (FISH) with HER2 positivity defined as IHC 3+ and/or FISH+ (*HER2*/CEN 17 ≥ 2.0). The study demonstrated the clinical utility of both HercepTest™ (IHC) and HER2 FISH pharmDx™ Kit (FISH) for the assessment of HER2 status in patients with advanced gastric or gastroesophageal junction adenocarcinoma for whom trastuzumab treatment is being considered (37, 38). Trastuzumab is a humanized monoclonal antibody that binds with high affinity to the HER2 protein and has been shown to inhibit the proliferation of human tumor cells that overexpress HER2 protein in vitro and in vivo (33-36).

Characteristics

In the BO18255 (ToGA) study, the HER2 status of all patients was determined for both HER2 protein overexpression by IHC (HercepTest™, Dako) and *HER2* gene amplification by FISH (*HER2* FISH pharmDx™ Kit, Dako) with HER2 positivity defined as IHC 3+ or FISH+ (*HER2*/CEN-17 ≥ 2.0). The results from this study showed that ~22% of the patients with advanced gastric or gastroesophageal junction adenocarcinoma were HER2-positive (38).

No patients were enrolled whose tumors were not gene amplified but HER2 protein weakly to strongly overexpressing [FISH(-)/IHC 2+] therefore it is unclear if patients whose tumors are not gene amplified but HER2 protein overexpressing [i.e., FISH(-), IHC 2+ or 3+] will benefit from Herceptin® treatment. The study also demonstrated that gene amplification (FISH) and protein overexpression (IHC) are not as correlated as with breast cancer, therefore a single method should not be used to determine HER2 status.

For use of the HercepTest™ in the assessment of patients for whom trastuzumab treatment is being considered, please see the package insert for Herceptin® for more information.

Principle of Procedure - Gastric

The HercepTest™ contains reagents required to complete a two-step immuno-cytochemical staining procedure for routinely processed, paraffin-embedded specimens. Following incubation with the primary rabbit 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 immunoglobulin molecules and horseradish peroxidase molecules linked to a common dextran polymer backbone, thus eliminating the need for sequential application of link antibody and peroxidase-conjugated antibody. Cross-reaction of the Visualization Reagent with human immunoglobulins and fetal calf serum has been removed by solid-phase absorption. 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. Results are interpreted using a light microscope.
Control Slides containing three formalin-fixed, paraffin-embedded human breast cancer cell lines with staining intensity scores of 0, 1+, and 3+ are provided to validate staining runs.

The staining intensity of these cell lines has been correlated to the number of receptors per cell.

HercepTest™, Code K5207, is applicable for automated staining using the Autostainer.

Reagents - Gastric

Materials provided

The materials listed below are sufficient for 50 tests (50 slides incubated with Primary Antibody to HER2 Protein and 50 slides incubated with the corresponding Negative Control Reagent). The number of tests is based on the use of 200 µL per slide of vials Nos. 1, 2, 3 and 4, and of the Substrate-Chromogen Solution (DAB). The kit provides materials sufficient for a maximum of 15 individual staining runs.

Vial No.	Quantity	Description
1	2 x 11 mL	PEROXIDASE-BLOCKING REAGENT Peroxidase-Blocking Reagent: 3% hydrogen peroxide containing 15 mmol/L sodium azide (NaN_3).
2	1 x 12 mL	RABBIT ANTI-HUMAN HER2 PROTEIN Rabbit Anti-Human HER2 Protein: Ready-to-use affinity-isolated antibody. Supplied in 0.05 mol/L Tris/HCl, 0.1 mol/L NaCl, 15 mmol/L NaN_3, pH 7.2, containing stabilizing protein. Immunogen: Synthetic C-terminal fragment (intracytoplasmic part) of the HER2 protein coupled to keyhole limpet hemocyanin. Specificity: HER2 protein. Purification method: The antibody is affinity isolated by using an immobilized HER2 protein peptide.
3	2 x 11 mL	VISUALIZATION REAGENT Visualization Reagent: Dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-rabbit immunoglobulins. Supplied in Tris/HCl buffer containing stabilizing protein and an antimicrobial agent.
4	1 x 12 mL	NEGATIVE CONTROL REAGENT Negative Control Reagent: Immunoglobulin fraction of normal rabbit serum at an equivalent protein concentration as the antibody to HER2 protein. Supplied in 0.05 mol/L Tris/HCl, 0.1 mol/L NaCl, 15 mmol/L NaN_3, pH 7.2, containing stabilizing protein.
5	15 x 11 mL	DAB BUFFERED SUBSTRATE DAB Buffered Substrate: Substrate buffer solution, pH 7.5, containing <0.1% hydrogen peroxide, stabilizers, enhancers, and an antimicrobial agent.
6	3 x 3 mL	DAB CHROMOGEN DAB Chromogen: 5% 3,3'-diaminobenzidine tetrahydrochloride chromogen solution.
7	1 x 1 L	EPITOPE RETRIEVAL SOLUTION (X 10) Epitope Retrieval Solution (x 10): 0.1 mol/L citrate buffer with an antimicrobial agent.

8	2 x 1 L	WASH BUFFER (10x) Wash Buffer (10x): Tris/HCl buffer with a detergent and an antimicrobial agent.
	3 x 5 slides	CONTROL SLIDES Control Slides: Each slide contains sections of three formalin-fixed, paraffin-embedded breast carcinoma cell lines representing different levels of HER2 protein expression: MDA-231 (0), MDA-175 (1+), and SK-BR-3 (3+). The Control Slides have been heat treated for better adherence of sections to glass slides. Any additional heat treatment of Control Slides performed to improve the adherence of sections to glass slides may compromise staining results.

NOTE: All reagents, including Epitope Retrieval Solution and Wash Buffer, are formulated specifically for use with this test. For the test to perform as specified, no substitutions should be made except for the Wash Buffer, where Dako Code S3006 may be used.

Materials required but not provided

Ammonium hydroxide, 15 mol/L diluted to 37 mmol/L

Counterstain: Hematoxylin, such as water-based Mayer's Hematoxylin, Dako Code S3301 (see INSTRUCTIONS FOR USE, A.4)

Coverslips

Distilled or deionized water (Washing Water)

Drying oven, capable of maintaining 60 °C or less

Ethanol, absolute and 95%

Light microscope (4–40x objective magnification)

Mounting medium, such as Dako Faramount, Code S3025, or Glycergel™, Code C0563

Positive and Negative Tissues to use as process controls (see Quality Control Section)

Slides, SuperFrost Plus, poly-L-lysine-coated slides, or Dako Silanized Slides, Code S3003, (see Specimen Preparation)

Staining jars or baths

Timer (capable of 2–40 minute intervals)

Water bath with lid (capable of maintaining Epitope Retrieval Solution at 95–99 °C)

Xylene, toluene, or xylene substitutes

K5207 has been tailored for use with the Autostainer Immunostaining System, Code S3400. Please refer to the Autostainer User Guide for necessary Autostainer Components.

Storage - Gastric

Store at 2–8 °C.

Do not use the kit after the expiration date stamped on the outside package. If reagents are stored under any conditions other than those specified in this package insert, they must be validated by the user (14a, 14b). Note that Control Slides must also be stored at 2–8 °C.

There are no obvious signs to indicate instability of this product. Therefore, Positive and Negative Controls should be run simultaneously with patient specimens. If unexpected staining is observed, which cannot be explained by variations in laboratory procedures, and a problem with HercepTest™ is suspected, immediately contact Dako's Technical Services.

Specimen Preparation - Gastric

Gastric or gastroesophageal junction adenocarcinoma specimens from biopsies, excisions, or resections must be handled correctly to preserve the tissue for immunocytochemical staining. Standard methods of tissue processing should be used for all specimens (15). When testing small biopsy specimens, ascertain intact tumor morphology and the presence of sufficient tumor cells for IHC evaluation. If HercepTest™ analysis is performed on a biopsy specimen, multiple (7-8) evaluable biopsy specimens from different regions of the tumor should be analysed to ensure reliable determination of HER2 status.

Paraffin-embedded sections

Tissues preserved in neutral buffered formalin and paraffin embedding are suitable for use. Specimens should e.g. be cut into blocks of a thickness of 3 or 4 mm and fixed for 18–24 hours in neutral-buffered formalin. Biopsy specimens were fixed for 6-8 hours in the ToGA trial (for study reference, refer to (37)). The tissues are then 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) (15, 16). In the USA, the Clinical Laboratory Improvement Act of 1988 requires in 42 CFR 493.1259(b) that "The laboratory must retain stained slides at least ten years from the date of examination and retain specimen blocks at least two years from the date of examination" (16).

Tissue specimens should be cut into sections of 4–5 µm, mounted onto slides and 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. **CAUTION:** Excessive heating for more than one hour at ≥60 °C may cause a significant decrease or loss of the specific membrane-associated HER2 immunoreactivity (17).

To preserve antigenicity, tissue sections mounted onto slides (SuperFrost Plus, poly-L-lysine or silanized slides) should be stained within 4-6 weeks of sectioning when stored at room temperature (20–25 °C) (18). The slides required for HER2 protein evaluation and verification of tumor presence should be prepared at the same time. A minimum of 5 slides is recommended, 1 slide for tumor presence, 2 slides for HER2 protein evaluation (1 for incubation with vial No. 2 and 1 for incubation with vial No. 4), and 2 slides for back-up.

The use of HercepTest™ on decalcified tissues has not been validated and is not recommended.

Consult Dako's "Education Guide: Immunohistochemical Staining Methods" (19) and references 15 and 16 for further details on specimen preparation.

Treatment of tissues prior to staining

A specific epitope retrieval method in 10 mmol/L citrate buffer, must be used for optimal assay performance. The Epitope Retrieval Solution is supplied in the HercepTest™ kit. This method involves heating of tissue sections mounted on slides that are immersed in 10 mmol/L citrate buffer (20) in a calibrated water bath capable of maintaining the Epitope Retrieval Solution at the required temperature (95–99 °C). Laboratories located at higher elevations should determine the best method of maintaining the required water bath temperature. The epitope retrieval must be performed in a water bath. Other methods of heating have been tested and do not give reproducible results. Immediately after epitope retrieval, commence the staining procedure. Deviation from the described procedure may affect results.

Precautions - Gastric

1. For in vitro diagnostic use.
2. For professional users.
3. Vial 1, Peroxidase-Blocking Reagent, contains 3% hydrogen peroxide. Safety data sheet available for professional users on request.

4. Vial 6, DAB Chromogen, contains 1-5% 3,3'-diaminobenzidine tetrahydrochloride (biphenyl-3,3',4,4'-tetrayltetraammonium tetrachloride) and is labeled:
   Harmful.
   R40 Limited evidence of a carcinogenic effect.
   R43 May cause sensitization by skin contact.
   R68 Possible risk of irreversible effects.
   S35 This material and its container must be disposed of in a safe way.
   S36/37 Wear suitable protective clothing and gloves.
   As a general rule, persons under 18 years of age are not allowed to work with this product. Users must be carefully instructed in the proper work procedure, the dangerous properties of the product and the necessary safety instructions (per European Union Directive 94/33/EC). Please refer to the Material Safety Data Sheet (MSDS) for additional information.

   USA: 3,3'-diaminobenzidine (DAB) may be harmful by inhalation, in contact with skin and if swallowed. Material is irritating to eyes and skin. If skin contact should occur, rinse affected areas with soap and water.

   NOTE: Although diaminobenzidine is structurally related to benzidine, there is no evidence for the carcinogenicity of diaminobenzidine. Consult Federal, State, or local regulations for disposal.

5. Vial 8, Wash Buffer, contains 5-bromo-5-nitro-1,3-dioxane that may produce an allergic reaction. Safety data sheet available for professional users on request.

6. This product contains sodium azide (NaN_3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, sodium azide may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing (21, 22).

7. Vial 2, 3 and 4 contain material of animal origin. As with any product derived from biological sources, proper handling procedures should be used.

8. Control slides and specimens, before and after fixation, and all materials exposed to them, should be handled as if capable of transmitting infection, and disposed of with proper precautions (23). Never pipette reagents by mouth and avoid contacting the skin and mucous membranes with reagents and specimens. If reagents come in contact with sensitive areas, wash with copious amounts of water.

9. Minimize microbial contamination of reagents to avoid non-specific staining.

10. Incubation times, temperatures, or methods other than those specified may give erroneous results. Excess drying at ≥60 °C for more than one hour may cause a significant decrease or loss of the specific membrane-associated HER2 immunoreactivity (17).

11. Reagents have been optimally diluted. Further dilution may result in loss of antigen staining.

12. All reagents, including Epitope Retrieval Solution and Wash Buffer, are formulated specifically for use with this test. In order for the test to perform as specified, no substitutions should be made except for the Wash Buffer, where Code S3006 may be used.

13. The Visualization Reagent and DAB Chromogen may be affected adversely if exposed to excessive light levels. Do not store system components or perform staining in strong light, such as direct sunlight.

14. Wear appropriate personal protective equipment to avoid contact with eyes and skin. Refer to the Material Safety Data Sheet (MSDS) for additional information.

15. For accurate interpretation of HercepTest™ results on stained biopsy samples from gastric or gastroesophageal junction adenocarcinoma, a cluster of at least 5 stained tumor cells is recommended.

16. Due to the heterogeneous nature of gastric cancer biopsy specimens it is important to perform HER2 IHC testing on multiple pieces (7-8) of biopsy from different regions of the tumor to obtain a reliable result.
17. HER2 protein overexpression and *HER2* gene amplification are not as well correlated in gastric cancer as with breast cancer, therefore a single method should not be used to determine HER2 status.

INSTRUCTIONS FOR USE - Gastric

A. Reagent Preparation

It is convenient to prepare the following reagents prior to staining:

A.1 Epitope Retrieval Solution

Dilute a sufficient quantity of Vial 7 (Epitope Retrieval Solution x 10) 1:10 using distilled or deionized water for the staining procedure that is planned. Unused diluted solution may be stored at 2–8 °C for one month. Discard diluted solution if cloudy in appearance.

A.2 Wash Buffer

Dilute a sufficient quantity of Vial 8 (Wash Buffer x 10) 1:10 using distilled or deionized water for the wash steps. Unused diluted buffer may be stored at 2–8 °C for one month. Discard buffer if cloudy in appearance.

The Autostainer is programmed to rinse tissue sections after the Peroxidase-Blocking Reagent and Substrate-Chromogen Solution. Note that for these rinse steps either distilled (or deionized) water or Wash Buffer may be used. All other rinse steps require the use of Wash Buffer.

A.3 Substrate-Chromogen Solution (DAB)

Prepare Substrate-Chromogen Solution by adding 11 drops (25–30 □L per drop) of DAB Chromogen from Vial 6 to one Vial 5 containing DAB Buffered Substrate (11 mL) and mix. Prepared Substrate-Chromogen Solution (DAB) is stable for approximately 5 days when stored at 2–8 °C. This solution should be mixed thoroughly prior to use. Any precipitate developing in the solution does not affect staining quality.

NOTE: The color of the DAB Chromogen in Vial 6 may vary from clear to light lavender-brown. This will not affect the performance of this product. Please dilute according to the guidelines in this package insert. Addition of excess DAB Chromogen to the DAB Buffered Substrate will result in deterioration of the positive signal.

A.4 Counterstain

The colored end-product of the DAB staining reaction is alcohol and water insoluble. Use a hematoxylin counterstain and adjust the hematoxylin staining intensity to a similar level shown in Dako's "Guidelines for Scoring HercepTest™- Gastric Cancer". Hematoxylin, either alcohol or water-based, such as Dako Mayer's Hematoxylin, Code S3301, may be used.
Follow hematoxylin counterstaining with a thorough rinse in distilled or deionized water, then immerse tissue slides into a bath of 37 mmol/L ammonia water (see Section B.2, Step 3). Ammonia water (37 mmol/L) is prepared by mixing 2.5 mL of 15 mol/L (concentrated) ammonium hydroxide with 1 liter of distilled or deionized water. Unused 37 mmol/L ammonia water may be stored at room temperature (20–25 °C) in a tightly capped bottle for up to 12 months.

A.5 Mounting Medium

Non-aqueous, permanent mounting medium is recommended. However, aqueous mounting is also acceptable. Dako Faramount Aqueous Mounting Medium, Ready-to-Use, Code S3025, or Glycergel™ Mounting Medium, Code C0563, is recommended for aqueous mounting. Liquefy Glycergel™ by warming to approximately 40 (± 5) °C prior to use.

B. Staining Procedure Performed on the Autostainer

B.1 Procedural notes

The user should read these instructions carefully and become familiar with all components and instrumentation prior to use (see Precautions).

All reagents should be equilibrated to room temperature (20–25 °C) prior to immunostaining. Likewise, all incubations should be performed at room temperature.

Do not allow tissue sections to dry while loading slides on the Autostainer and during the staining procedure. Dried tissue sections may display increased non-specific staining.

If the staining procedure is interrupted, slides may be kept in a buffer bath following incubation of the primary antibody for up to one hour at room temperature (20–25 °C) without affecting the staining performance.

Deparaffinization and rehydration: Prior to staining, tissue slides must be deparaffinized to remove embedding medium and rehydrated. Avoid incomplete removal of paraffin. Residual embedding medium will result in increased non-specific staining. This step should be performed at room temperature (20–25 °C).

1. Place slides in a xylene bath and incubate for 5 (±1) minutes. Change baths and repeat once.
2. Tap off excess liquid and place slides in absolute ethanol for 3 (±1) minutes. Change baths and repeat once.
3. Tap off excess liquid and place slides in 95% ethanol for 3 (±1) minutes. Change baths and repeat once.
4. Tap off excess liquid and place slides in distilled or deionized water for a minimum of 30 seconds. Commence staining procedure as outlined in Section B.2, Step 1, Epitope retrieval.

Xylene and alcohol solutions should be changed after 40 slides. Toluene or xylene substitutes, such as Histoclear, may be used in place of xylene.

NOTE: The reagents and instructions supplied in this kit have been designed for optimal performance. Further dilution of the reagents or alteration of incubation temperatures may give erroneous or discordant results. Differences in tissue processing and technical procedures in the user's laboratory may invalidate the assay results for use in selecting patients for Herceptin® therapy.

B.2 Staining protocol

Performed at room temperature, 20–25 °C.

Step 1: Epitope retrieval

Fill staining jars, e.g. Coplin jars, with the diluted Epitope Retrieval Solution (see INSTRUCTIONS FOR USE, Section A.1). Place staining jars containing Epitope Retrieval Solution in water bath. Heat water bath and the Epitope Retrieval Solution to 95–99 °C. Cover jars with lids to stabilize the temperature and avoid evaporation.

Immerse the room temperature deparaffinized sections in the preheated Epitope Retrieval Solution in the staining jars. **BRING TEMPERATURE OF THE WATER BATH AND THE EPITOPE RETRIEVAL SOLUTION BACK TO 95–99 °C.** Incubate for 40 (±1) minutes at 95–99 °C.

Remove the entire jar with slides from the water bath. Allow the slides to cool in the Epitope Retrieval Solution for 20 (±1) minutes at room temperature.

Decant the Epitope Retrieval Solution and rinse sections in the Wash Buffer (see INSTRUCTIONS FOR USE, Section A.2).

For optimal performance, soak sections in Wash Buffer for 5–20 minutes after epitope retrieval and prior to staining.

NOTE: The Epitope Retrieval Solution is designed for single use application only. Do not re-use.

Step 2: Autostainer procedure

1. Use the Autostainer-generated map on the HercepTest™ autoprogram for required program times and reagent volumes (see point 4 below for specific volumes).
2. Place the Autostainer reagent vials in the Autostainer reagent rack according to the computer-generated reagent map.
3. Load the slides onto the Autostainer according to the computer- generated slide map.
4. Set Program and begin the HercepTest™ program. The following is an outline of the program run:
   - Rinse
   - 200 µL Peroxidase-Blocking Reagent - 5 minutes
   - Rinse
   - 200 µL Primary Anti-HER2 Protein (or Negative Control Reagent) - 30 minutes
   - Rinse
   - 200 µL Visualization Reagent - 30 minutes
   - Rinse
   - Rinse
   - Switch
   - 200 µL Substrate-Chromogen Solution (DAB) - 10 minutes

Rinse slides in deionized water after the substrate-chromogen step

NOTE: Autostainer Hardware version 01 rinses slides in buffer. Therefore, the slides must be rinsed with deionized water after they have been removed from the Autostainer.

Step 3: Counterstain (instructions are for hematoxylin)

Remove slides from the Autostainer and counterstain in hematoxylin as described below.

Immerse slides in a bath of hematoxylin. Incubate for 2–5 minutes, depending on the strength of hematoxylin used.

Rinse *gently* in a distilled or deionized water bath. Ensure all residual hematoxylin has been cleared.

Optional: Dip slides 10 times into a bath of 37 mmol/L ammonia water (see Section A.4).

Rinse slides *gently* in a bath of distilled or deionized water for 2–5 minutes.

NOTE: Depending on the incubation length and potency of the hematoxylin used, counterstaining will result in a pale to dark blue coloration of the cell nuclei. Excessive or incomplete counterstaining may compromise proper interpretation of results.

Step 4: Mounting

Non-aqueous, permanent mounting medium is recommended. Otherwise, aqueous mounting medium is also acceptable. Specimens may be mounted and coverslipped with a water-based mounting medium such as Dako Faramount, Code S3025, or Glycergel™, Code C0563.

NOTE: Slides may be read when convenient. However, some fading may occur if slides are coverslipped with an aqueous mounting medium and exposed to strong light over a period of one week. To minimize fading, store slides in the dark at room temperature (20–25 °C).

Quality Control - Gastric

Differences in tissue fixation, processing and embedding in the user's laboratory may produce significant variability in results, necessitating regular performance of in-house controls in addition to the Control Slides supplied by Dako. In the USA, consult the quality control guidelines of the College of American Pathologists (CAP) Certification Program for Immunohistochemistry, see also CLSI (formerly NCCLS) Quality Assurance for Immunocytochemistry, Approved Guideline (24), and reference 25 for additional information.

Table 8. The purpose of daily quality control.

Tissue: Fixed and Processed Like Patient Sample	Specific Antibody and Secondary Antibody	Non-Specific Antibody* or Buffer Plus Same Secondary Antibody as Used with Specific Antibody
Positive Control: Tissue or cells containing target antigen to be detected (could be located in patient tissue). The ideal control is weakly positive staining tissue as this is most sensitive to antibody or antigen degradation	Controls all steps of the analysis. Validates reagent and procedures used for HER2 protein staining	Detection of non-specific background staining
Negative Control: Tissues or cells expected to be negative (could be located in patient tissue or positive control tissue)	Detection of unintended antibody cross-reactivity to cells/cellular components	Detection of non-specific background staining
Patient tissue	Detection of specific staining	Detection of non-specific background staining
Control Slide supplied by Dako	Controls staining procedure only	

* Serum from same species as the specific antibody, but not directed against the same target antigen. To detect non-specific antibody binding, e.g. binding of Fc portion of antibody by the tissue.

Control Slide (provided): Each of the supplied Control Slides contains three pelleted, formalin-fixed, paraffin-embedded human breast cancer cell lines with staining intensity scores of 0, 1+ and 3+. One slide should be stained in each staining run. The evaluation of the Control Slide cell lines supplied by Dako indicates the validity of the staining run.

Positive Control Tissue: Controls should be fresh autopsy/biopsy/surgical specimens fixed, processed and embedded as soon as possible in the same manner as the patient sample(s). Positive tissue controls are indicative of correctly prepared tissues and proper staining techniques. One Positive Control Tissue for each set of test conditions should be included in each staining run.

The Positive Control Tissues should give weak positive staining so they can detect subtle changes in the primary antibody sensitivity. The Control Slides supplied with this kit or specimens processed differently from the patient sample(s) validate reagent performance only and do not verify tissue preparation. Use previously determined HER2 protein 2+ overexpressing tissue from metastatic gastric or gastroesophageal junction adenocarcinoma, for the ideal Positive Control Tissue.

NOTE: Known positive control tissue should only be utilized for monitoring the correct performance of processed tissues and test reagents, NOT as an aid in formulating a specific

diagnosis of patient samples. If the Positive Control Tissue fails to demonstrate appropriate positive staining, results with the patient specimens should be considered invalid.

Negative Control Tissue: Use a negative control tissue (known to be HER2 protein negative) fixed, processed and embedded in a manner identical to the patient sample(s) with each staining run to verify the specificity of the primary antibody and to provide an indication of specific background staining. Colon, liver or thyroid are appropriate for negative control tissue. The variety of different cell types present in most tissue sections offers internal negative control sites (this should be verified by the user).

If specific staining occurs in the Negative Control Tissue, results with the patient specimens should be considered invalid and the test re-run.

Non-Specific Negative Control Reagent: Use the supplied Negative Control Reagent in place of the primary antibody with a section of each patient specimen to evaluate non-specific staining and allow better interpretation of specific staining at the antigen site. The incubation period for the Negative Control Reagent should correspond to that of the primary antibody.

Assay verification: Prior to initial use of an antibody or staining system in a diagnostic procedure, the user should verify the antibody's specificity by testing it on a series of in-house tissues with known immunocytochemical performance characteristics representing known positive and negative tissues. Refer to the quality control procedures previously outlined in this section of the product insert and to the quality control requirements of the CAP Certification Program for Immunohistochemistry and/or CLSI (formerly NCCLS) Quality Assurance for Immunocytochemistry, Approved Guideline (24). These quality control procedures should be repeated for each new antibody lot, or whenever there is a change in assay parameters. Gastric or gastroesophageal junction adenocarcinoma, with known HER2 protein staining intensities from 0 - 3+ and negative tissues, e.g. colon, liver or thyroid are suitable for assay verification.

Interpretation of Staining - Gastric

For determination of HER2 protein expression, only the membrane staining intensity and pattern should be evaluated using the scale presented in Table 9. Slide evaluation should be performed by a pathologist using a light microscope. For evaluation of the immunohistochemical staining and scoring, an objective of 10X magnification is appropriate. The use of a 5–40x objective magnification is useful in confirmation of the score. Cytoplasmic staining should be considered non-specific staining and is not to be included in the assessment of membrane staining intensity (8). To aid in the differentiation of 0, 1+, 2+ and 3+ staining, refer to Dako's "Guidelines for Scoring HercepTest™ – Gastric" for representative pictures of the staining intensitites and patterns.

Only specimens from patients with gastric or gastroesophageal junction adenocarcinoma, should be scored. In cases with intestinal metaplasia and adenocarcinoma in the same specimen, only the adenocarcinoma component should be scored. For interpretation of HercepTest™ stained biopsies a cluster of at least 5 stained tumor cells is recommended.

Table 9. Interpretation and scoring of HER2 immunohistochemical staining

Score	Surgical Specimen – Staining Pattern	Biopsy Specimen – Staining Pattern	HER2 Overexpression Assessment
0	No reactivity or membranous reactivity in < 10% of tumor cells	No reactivity or no membranous reactivity in any (or < 5 clustered) tumor cell	Negative
1+	Faint/barely perceptible membranous reactivity in ≥ 10% of tumor cells; cells are reactive only in part of their membrane	Tumor cell cluster (≥ 5 cells) with a faint/barely perceptible membranous reactivity irrespective of percentage of tumor cells stained	Negative
2+	Weak to moderate complete, basolateral or lateral membranous reactivity in ≥ 10% of tumor cells	Tumor cell cluster (≥ 5 cells) with a weak to moderate complete, basolateral or lateral membranous reactivity irrespective of percentage of tumor cells stained	Equivocal
3+	Strong complete, basolateral or lateral membranous reactivity in ≥ 10% of tumor cells	Tumor cell cluster (≥ 5 cells) with a strong complete, basolateral or lateral membranous reactivity irrespective of percentage of tumor cells stained	Positive

Guidelines based on Hofmann et al. (39).

HercepTest™ is interpreted as negative for HER2 protein overexpression (0 and 1+ score), equivocal (2+ score), and positive (3+ score). HercepTest™ is not intended to provide prognostic information to the patient and physician and has not been validated for that purpose.

For each staining run, slides should be examined in the order presented in Table 10 to determine the validity of the staining run and enable semi-quantitative assessment of the staining intensity of the sample tissue.

Table 10. Order of slide evaluation.

Slide Reading Order	Rationale
1. Control Slide containing the three cell lines	Presence of 3+ brown cell membrane staining (rimming) in the 3+ Control Cell Line SK-BR-3, partial brown rimming in the 1+ Control Cell Line MDA-175, and no staining in the 0 Control Cell Line MDA-231 indicates a valid assay Punctate and discontinuous membrane staining is present in a small to moderate number of the cells in the weakly positive 1+ Control Cell Line MDA-175. Also dot-like immunostaining of the Golgi region of the cytoplasm can be observed in this cell line Presence of brown staining in the 0 Control Cell Line MDA-231 (negative for HER2 protein staining) indicates that there was non-specific staining during the assay. The assay results may be invalid due to overstaining
2. Positive Control Tissue Slide	Presence of brown membrane staining should be observed. Staining of the cytoplasm and negative tissues should not be more than 1+
3. Negative Control Tissue Slide	The **ABSENCE** of specific staining in the Negative Control Tissue Slide confirms the lack of kit cross-reactivity to cells/cellular components. If specific membrane staining occurs in the Negative Control Tissue Slide, results with the patient specimen should be considered invalid
4. Patient tissue slide stained using the Negative Control Reagent	Absence of specific membrane staining verifies the specific labeling of the target antigen by the primary antibody Other tan or brown staining occurring in the cytoplasm of the specimen treated with the Negative Control Reagent, such as in connective tissue, leukocytes, erythrocytes, or necrotic tissue, should be considered non-specific background staining and should be reported under the comments section of the data spreadsheet
5. Patient tissue slide stained using the primary antibody	When HER2 protein overexpression is detected in the specimen, it will appear as brown rimming localized on the cell membrane of tumor cells treated with the primary antibody

1. Control Slide (provided): The Control Slide stained with HercepTest™ should be examined first to ascertain that all reagents are functioning properly. The presence of a brown (3,3'-diaminobenzidine, DAB) reaction product at the cell membrane is indicative of positive reactivity.

Presence of circumferential brown cell membrane staining (rimming) in the 3+ Control Cell Line SK-BR-3, partial brown rimming in the 1+ Control Cell Line MDA-175, and no staining in the 0 Control Cell Line MDA-231 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.

2. Positive Control Tissue: The Positive Control Tissue Slide should be examined next. This slide verifies that the fixation method and epitope retrieval process are effective. Use intact cells for interpretation of staining results because necrotic or degenerated cells often stain non-specifically (26). Staining should be observed in tumor tissue as brown, cell membrane staining. Brown staining of the cytoplasm and negative tissues within the specimen should not be more than corresponding to 1+ staining intensity score.

3. Negative Control Tissue: The Negative Control Tissue Slide should be examined after the Positive Control Tissue to verify the specificity of the labeling of the target antigen by the primary antibody. The absence of specific staining in the Negative Control Tissue confirms the lack of kit cross-reactivity to cells/cellular components. If specific staining occurs in the

Negative Control Tissue, results with the patient specimen should be considered invalid. Alternatively, negative portions of the Positive Control Tissue may serve as the Negative Control Tissue, but this should be verified by the user. Note that a weak reaction (0 - 1+ staining intensity) can be observed in most normal epithelial tissue. Possible negative control tissues include: colon, liver and thyroid.

Non-specific staining, if present, will be of a diffuse appearance. Sporadic staining of connective tissue may also be observed in sections from excessively formalin-fixed tissues.

4 + 5. Patient Tissue: Examine patient specimens stained with HercepTest™ last. Positive staining intensity should be assessed within the context of any non-specific background staining of the Negative Control Reagent. As with any immunocytochemical test, a negative result means that the antigen was not detected, not that the antigen was absent in the cells/tissue assayed. Refer to Summary and Explanation, Limitations, and Performance Characteristics for specific information regarding HercepTest™ immunoreactivity.

Additional Recommendations for Interpretation of HercepTest™ Staining
Gastric or gastroesophageal junction adenocarcinoma tested for HER2 protein overexpression are scored from 0 to 3+. While the 0 and 3+ cases are clear-cut, a small percentage of the remaining 1+ and 2+ samples may be more difficult to interpret. Use the following guidelines for interpretation of HercepTest™ staining in your laboratory.

- Evaluate the Control Cell Lines to validate the assay performance.
- Evaluate the Positive and Negative Control Slides.
- A hematoxylin and eosin (H&E) staining 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 is required from the pathologist to verify the presence of the tumor). The HercepTest™ should be performed on a paired section (serial section) from the same paraffin block of the specimen.
- Evaluate the sections stained for HER2 protein overexpression at low power first. The majority of positive cases will be obvious at low power magnification.
- For 1+ cases, use 40x objective magnification to verify membrane staining.
- For 2+ cases, use 10x-20x objective magnification to verify membrane staining.

Surgical specimen

- Well-preserved and well-stained areas of the specimen should be used to make a determination of the percent of positive stained tumor cells.
- If a majority of tumor cells demonstrate complete, basolateral or lateral membrane staining, the staining is either 2+ or 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+.
- 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+.
- 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+.
- If no staining is observed the score of the surgical specimen is 0.
- 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

- 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.
- 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.
- 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.
- If no staining is observed the score of the biopsy specimen is 0.
- If membrane staining (irrespective of staining intensity) is observed in less than 5 clustered tumor cells, the score of the biopsy specimen is 0.

Limitations - Gastric

General limitations

1. Immunocytochemistry is a multi-step diagnostic process that requires specialized training in the selection of the appropriate reagents; tissue selection, fixation, and processing; preparation of the immunocytochemistry slide; and interpretation of the staining results.
2. Tissue staining is dependent on the handling and processing of the tissue prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, sectioning, or contamination with other tissues or fluids may produce artifacts, antibody trapping, or false-negative results. Inconsistent results may be due to variations in fixation and embedding methods, or to inherent irregularities within the tissue.
3. Excessive or incomplete counterstaining may compromise proper interpretation of results.
4. The clinical interpretation of any positive staining or its absence must be evaluated within the context of clinical presentation, morphology and other histopathological criteria. The clinical interpretation of any staining, or its absence, must be complemented by morphological studies and proper controls as well as other diagnostic tests. It is the responsibility of a qualified pathologist, who is familiar with the antibodies, reagents and methods used, to interpret the stained preparation. Staining must be performed in a certified, licensed laboratory under the supervision of a pathologist who is responsible for reviewing the stained slides and assuring the adequacy of positive and negative controls.
5. Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit non-specific staining with horseradish peroxidase (27). Reagents may demonstrate unexpected reactions in previously untested tissue types. The possibility of unexpected reactions even in tested tissue types cannot be completely eliminated due to biological variability of antigen expression in neoplasms, or other pathological tissues (28). Contact Dako's Technical Services with documented unexpected reaction.
6. False-positive results may be seen due to non-immunological binding of proteins or substrate reaction products. They may also be caused by pseudoperoxidase activity (erythrocytes) and endogenous peroxidase activity (cytochrome C) (28).
7. The staining procedure should be performed at ambient temperature of 20–25 °C.

Product-specific limitations

1. The antigen present in the 1+ Control Cell Line MDA-175 is subject to degradation over time. Assess the Control Slide results in connection with the expiration date of the Control Slide. Negative staining of the MDA-175 cells may only indicate that the Control Slide has degraded. The Control Slides must be stored at 2–8 °C.
2. False-negative results could be caused by degradation of the antigen in the tissues over time. Specimens should be stained within 4-6 weeks of mounting of tissues on slides when stored at room temperature (20–25 °C) (29).
3. For optimal and reproducible results, the HER2 protein requires heat-induced epitope retrieval when tissues are routinely fixed (neutral-buffered formalin) and paraffin embedded. This pre-treatment needs to be completed at the beginning of the entire staining process. See the "Specimen Preparation Section, Treatment of tissues prior to staining" for instructions.
4. Heat-induced epitope retrieval of the HER2 protein should only be done using a calibrated water bath. Other methods of heating have been tested and do not give reproducible results.
5. Do not replace kit reagents with reagents carrying other lot numbers or with reagents from other manufacturers. The only exception is the Wash Buffer that may be replaced with Dako Wash Buffer, Code S3006.
6. False results could be obtained from evaluation of cytoplasmic staining. Consider only the intensity of cell membrane staining when interpreting results.
7. Stained Control Slides should be used only for validation of the staining run and should *not* be used as a guide to score the staining reaction in tissue sections.
8. Strong focal staining (3+), i.e. "hot spots," may occasionally be seen. This may be the result of uneven fixation and/or processing of tissue. Immunostaining of a second tissue block from the same specimen is recommended.
9. Use of HercepTest™ on specimens fixed in fixatives other than neutral buffered formalin has not been validated.
10. Note that normal tonsil and esophageal epithelia may stain up to 2+ intensity.
11. Use of crushed gastric cancer specimens and interpretation of artifactual staining around a biopsy edge should be avoided.

Performance Characteristics - Gastric

Background

The clinical safety and efficacy of trastuzumab (Herceptin®) has been demonstrated in the BO18255 study (the ToGA trial) (36, 37). The study was designed as an open labeled, randomized, multicenter, phase III study in HER2-positive patients with advanced gastric or gastroesophageal junction adenocarcinoma. In this study HER2 positivity was defined as being either IHC-positive (3+) (HercepTest™, Dako) or positive by *HER2* FISH (*HER2*/CEN-17 ≥ 2.0) (*HER2* FISH pharmDx™ Kit, Dako). After inclusion in the study the patients were randomized to receive chemotherapy [cisplatin and a fluoropyrimidine (capecitabine or 5 fluorouracil); FC] or chemotherapy plus trastuzumab (FC+H).

In the BO18255 study a total of 594 patients were enrolled, of whom 594 (296 patients in the chemotherapy arm, 298 patients in the chemotherapy plus trastuzumab arm) received study medication.

The main efficacy outcome measure was overall survival (OS) analyzed by stratified log rank test. The final OS analysis based on 351 deaths was statistically significant (nominal significance level of 0.0193). An updated OS analysis was also conducted at one year after the final analysis. The median overall survival of 13.5 months on the Herceptin® plus

chemotherapy arm was significantly longer compared to 11.0 month median overall survival on the chemotherapy alone arm. The efficacy results of both the definitive and the updated (final) analyses are summarized in Table 11 and Figure 1.

Table 11. Overall Survival in Intention to Treat (ITT) Population

	FC Arm N= 296	FC + H Arm N=298
Final Overall Survival		
No. Deaths (%)	184 (62.2%)	167 (56.0%)
Median	11.0	13.5
95% CI (mos.)	(9.4, 12.5)	(11.7, 15.7)
Hazard Ratio	0.73	
95% CI	(0.60, 0.91)	
p-value*, two-sided	0.0038*	
Updated Overall Survival		
No. Deaths (%)	227 (76.7%)	221 (74.2%)
Median	11.7	13.1
95% CI (mos.)	(10.3, 13.0)	(11.9, 15.1)
Hazard Ratio	0.80	
95% CI	(0.67, 0.97)	

* Comparing with the nominal significance level of 0.0193

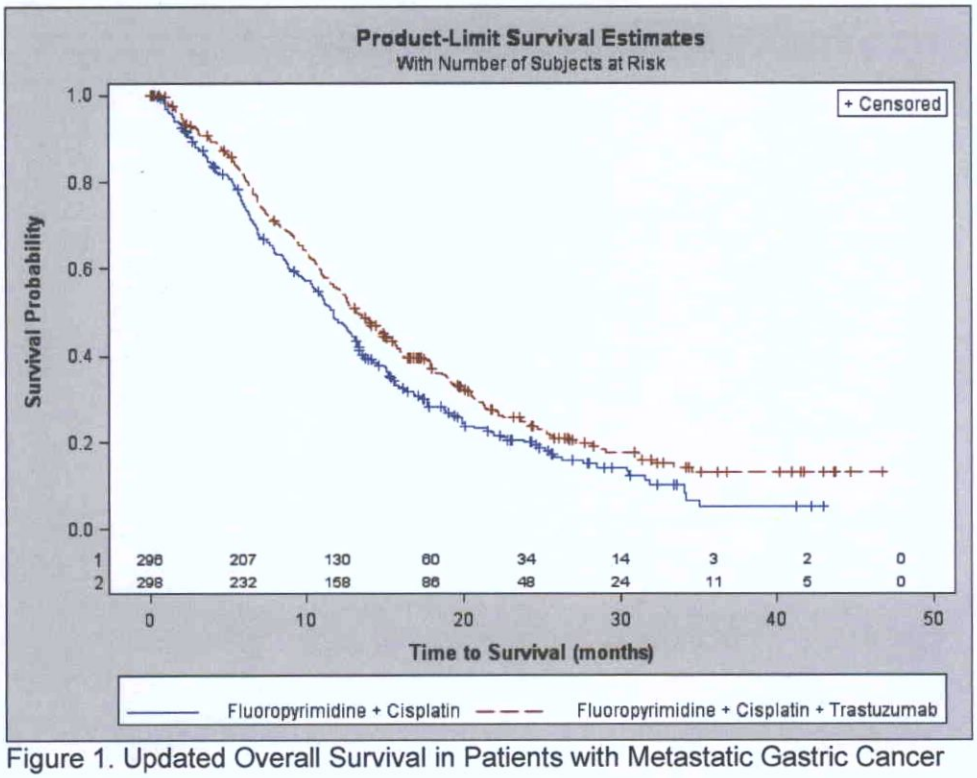

Figure 1. Updated Overall Survival in Patients with Metastatic Gastric Cancer

An exploratory analysis of OS based on gene amplification (FISH) and protein overexpression (IHC) testing is summarized in Table 12.

Table 12. Exploratory Analyses by HER2 Status Using the Updated Overall Survival Results.

	FC N=296[a]	FC+H N=298[b]
FISH+ / IHC 0, 1+ subgroup (N=133)		
No. Deaths / n (%)	57/71 (80.3%)	56/62 (90.3%)
Median OS Duration (mos.)	8.8	8.3
95% CI (mos.)	(6.4, 11.7)	(6.2, 10.7)
Hazard ratio (95% CI)	1.33 (092, 1.92)	
FISH+ / IHC2+ subgroup (N=160)		
No. Deaths / n (%)	65/80 (81%)	64/80 (80%)
Median OS Duration (mos.)	10.8	12.3
95% CI (mos.)	(6.8, 12.8)	(9.5, 15.7)
Hazard ratio (95% CI)	0.78 (0.55, 1.10)	
FISH+ or FISH-/IHC3+[c] subgroup (N=294)		
No. Deaths / n (%)	104/143 (73%)	96/151 (64%)
Median OS Duration (mos.)	13.2	18.0
95% CI (mos.)	(11.5. 15.2)	(15.5, 21.2)
Hazard ratio (95% CI)	0.66 (0.5, 0.87)	

Median survival was estimated from Kaplan-Meier curves.

[a] Two patients on FC arm who were FISH+ but IHC status unknown were excluded from the analyses.

[b] Five patients on Herceptin® arm who were FISH+ but IHC status unknown were excluded from the analyses.

[c] Includes 6 patients on chemotherapy arm, 10 patients on Herceptin® arm with FISH-, IHC3+ and 8 patients on chemotherapy arm, 8 patients on Herceptin® arm with FISH status unknown, IHC3+.

Reproducibility

HercepTest™ analysis of 60 different gastric cancer specimens obtained from stomach or gastroesophageal junction areas representing surgical resections and biopsies were performed on five non-consecutive days at three study sites. The 60 specimens in the study were equally distributed in the three HER2 status categories. A total of 2040 HER2 scorings were performed by six pathologists.

Day-to-day agreement (negative, equivocal, positive) ranged from 83.1% to 98.3%. In 47 of 60 possible comparisons the agreements were at 90.0% or above. In Table 13, specific examples of the day-to-day comparisons are shown with average agreements at 91.2%, 92.5% and 92.5% for the three sites.

Site-to-site agreements were 82.7%, 75.0% and 88.0%, respectively for pairwise site comparison (see Table 14). According to Fisher's exact test; the results were not different between sites.

Observer-to-observer agreements between pathologists at each site were 88.0%, 83.6% and 81.0% for the three study sites, respectively (Table 15).

In conclusion HercepTest™ analysis of gastric cancer specimens at three study sites demonstrated good agreement between observations with respect to days, sites and observers.

Table 13. Day-to-Day Overall Percent Agreements - A Subset of 12 out of 60 comparisons

		Observer 1		Observer 2		Average Agreement
		Agreement	CI95 LL[1]	Agreement	CI95 LL[1]	
Site 1	Day 1 vs Day 2	85.0	74.4	93.3	84.9	91.2
	Day 3 vs Day 4	93.3	84.9	93.3	84.9	
Site 2	Day 1 vs Day 2	95.0	87.3	83.1	72.0	92.5
	Day 3 vs Day 4	96.7	89.7	95.0	87.3	
Site 3	Day 1 vs Day 2	90.0	80.5	91.7	82.7	92.5
	Day 3 vs Day 4	96.7	89.7	91.7	82.7	

[1]CI95 LL: 95% lower limit confidence interval.

Table 14. Site-to-site Overall Percent Agreements

		Agreement	CI95 LL[1]	Average Agreement
Site 1 vs Site 2	Day 1 vs Day 1	83.3	72.4	82.7
	Day 2 vs Day 2	85.0	74.4	
	Day 3 vs Day 3	85.0	74.4	
	Day 4 vs Day 4	81.7	70.5	
	Day 5 vs Day 5	78.3	66.7	
Site 1 vs Site 3	Day 1 vs Day 1	80.0	68.6	75.0
	Day 2 vs Day 2	73.3	61.2	
	Day 3 vs Day 3	78.3	66.7	
	Day 4 vs Day 4	68.3	55.9	
	Day 5 vs Day 5	75.0	63.0	
Site 2 vs Site 3	Day 1 vs Day 1	88.3	78.5	88.0
	Day 2 vs Day 2	86.7	76.4	
	Day 3 vs Day 3	90.0	80.5	
	Day 4 vs Day 4	86.7	76.4	
	Day 5 vs Day 5	88.3	78.5	

[1]CI95 LL: 95% lower limit confidence interval.

Table 15. Observer-to-Observer Agreements

		Agreement	CI 95 LL[1]	Average Agreement
Site 1	Day 1	91.7	82.7	88.0
	Day 2	91.7	82.7	
	Day 3	93.3	84.9	
	Day 4	83.3	72.4	
	Day 5	80.0	68.6	
Site 2	Day 1	86.4	76.0	83.6
	Day 2	83.3	72.4	
	Day 3	83.3	72.4	
	Day 4	83.3	72.4	
	Day 5	81.7	70.5	
Site 3	Day 1	80.0	68.6	81.0
	Day 2	78.3	66.7	
	Day 3	80.0	68.6	
	Day 4	78.3	66.7	
	Day 5	90.0	80.5	

[1]CI95 LL: 95% lower limit confidence interval.

Immunoreactivity

Table 16 summarizes HercepTest™ immunoreactivity with the recommended panel of normal tissues. All tissues were formalin-fixed and paraffin-embedded and stained with HercepTest™ according to the instructions in the package insert.

Table 16. Summary of HercepTest™ normal tissue reactivity.

Tissue Type (No. Tested)	Positive Tissue Element Staining and Staining Pattern
Adrenal (3)	None
Bone marrow (3)	None
Brain/Cerebellum (3)	None
Brain/Cerebrum (3)	None
Breast (3)	Mammary gland (1+ staining intensity)
Cervix uteri (3)	None
Colon (3)	Columnar epithelium, surface (1+ staining intensity)
Esophagus (3)	Squamous epithelia (1/3 tissues, 2+ staining intensity)
Heart (3)	None
Kidney (3)	Tubule (1+ staining intensity)
Liver (3)	None
Lung (3)	None
Mesothelial cells (3)	None
Ovary (3)	None
Pancreas (3)	Langerhans cells, cytoplasmic (3+ staining intensity)
Parathyroid (3)	None
Peripheral nerve (3)	None
Pituitary (3)	Endocrine cells, cytoplasmic (3+ staining intensity)
Prostate (3)	Prostate gland (2+ staining intensity)
Salivary gland (3)	None
Skeletal muscle (3)	None
Skin (3)	None
Small intestine (3)	Columnar epithelium, surface (1+ staining intensity)
Spleen (3)	None
Stomach (3)	Epithelium (1/3 tissues, 1+ staining intensity)
Testis (3)	None
Thymus (3)	None
Thyroid (3)	None
Tonsil (3)	Squamous epithelia (2+ staining intensity)
Uterus (3)	Endometrium (1/3 tissues, 1+ staining intensity)

Reported staining in all tissues was membrane, unless otherwise noted. All three specimens of each tissue type had the same staining intensity unless otherwise noted.

Troubleshooting - Gastric

Refer to the Troubleshooting section in Dako's previously referenced Handbook (19) for remedial action, or contact Dako's Technical Service Department to report unusual staining.

Problem	Probable Cause	Suggested Action
1. No staining of slides	1a. Programming error. Reagents not used in proper order	1a. 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	1b. Check the Reagent Map to verify the proper location of reagent vials
	1c. Insufficient reagent in reagent vial	1c. Ensure that enough reagent is loaded into the reagent vials prior to commencing the run. Refer to Reagent Map for volumes required
	1d. Sodium azide in Wash Solution	1d. Use fresh preparation of Wash Buffer provided in the kit
	1e. Excessive heating of mounted tissue sections prior to deparaffinization and heat-induced antigen retrieval may lead to loss of visible HER2 immunoreactivity.	1e. 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 (17).
2. Weak staining of slides	2a. Inadequate epitope retrieval	2a. Verify that Epitope Retrieval Solution reaches 95–99 °C for a full 40 minutes and is allowed to cool for an additional 20 minutes
	2b. Inadequate reagent incubation times	2b. Review Staining Procedure instructions
	2c. Inappropriate fixation method used	2c. Ensure that patient tissue is not over-fixed or that an alternative fixative was not used
	2d. Excessive heating of mounted tissue sections prior to deparaffinization and heat-induced antigen retrieval may cause a significant decrease in visible HER2 immunoreactivity	2d. 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 (17).
3. Excessive background staining of slides	3a. Paraffin incompletely removed	3a. Use fresh clearing solutions and follow procedure as outlined in Section B.1
	3b. Starch additives used in	3b. Avoid using starch additives for

	mounting sections to slides	adhering sections to glass slides. Many additives are immuno-reactive
	3c. Slides not thoroughly rinsed	3c. 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
	3d. Sections dried during staining procedure	3d. 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
	3e. Sections dried while loading the Autostainer	3e. Ensure sections remain wet with buffer while loading and prior to initiating run
	3f. Inappropriate fixation method used	3f. Ensure that approved fixative was used. Alternative fixative may cause excessive back-ground staining
	3g. Non-specific binding of reagents to tissue	3g. Check fixation method of the specimen and presence of necrosis
4. Tissue detaches from slides	4a. Use of incorrect slides	4a. 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	5a 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	5b. Ensure that only a water bath is used for the epitope retrieval step
	5c. Reagent incubation times too long	5c. Review Staining Procedure instructions
	5d. Inappropriate wash solution used	5d. Use only the Wash Buffer that is recommended for the kit
6. Weak staining of the 1+ Control Slide Cell Line	6a. Incorrect epitope retrieval protocol followed	6a. 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)	6b. 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	6c. Check kit expiration date and kit storage conditions on outside of package

NOTE: If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please call Dako's Technical Services for further assistance.

Additional information on staining techniques and specimen preparation can be found in Dako's previously referenced Handbook (19) (available from Dako), Atlas of Immunohistology (30) and Immunoperoxidase Techniques. A Practical Approach to Tumor Diagnosis (31).

References

1. Coussens L, Yang-Feng TL, Liao Y-C, Chen E, Gray A, McGrath J, et al. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science 1985;230:1132-9.
2. King CR, Kraus MH, Aaronson SA. Amplification of a novel v-*erb*B-related gene in a human mammary carcinoma. Science 1985;229:974-6.
3. Semba K, Kamata N, Toyoshima K, Yamamoto T. A v-*erbB*-related protooncogene, c-*erbB-2*, is distinct from the c-*erbB-1*/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma. Proc Natl Acad Sci USA 1985;82:6497-501.
4. Yamamoto T, Ikawa S, Akiyama T, Semba K, Nomura N, Miyajima N, et al. Similarity of protein encoded by the human c-*erb-B*-2 gene to epidermal growth factor receptor. Nature 1986;319:230-4.
5. Schechter AL, Hung M-C, Vaidyanathan L, Weinberg RA, Yang-Feng TL, Francke U, et al. The *neu* gene: an *erbB*-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. Science 1985;229:976-8.
6. Bargmann CI, Hung M-C, Weinberg RA. The *neu* oncogene encodes an epidermal growth factor receptor-related protein. Nature 1986;319:226-30.
7. Natali PG, Nicotra MR, Bigotti A, Venturo I, Slamon DJ, Fendly BM, et al. Expression of the p185 encoded by HER2 oncogene in normal and transformed human tissues. Int J Cancer 1990;45:457-61.
8. Press MF, Cordon-Cardo C, Slamon DJ. Expression of the HER-2/*neu* proto-oncogene in normal human adult and fetal tissues. Oncogene 1990;5:953-62.
9. Lonardo F, Di Marco E, King CR, Pierce JH, Segatto O, Aaronson SA, et al. The normal *erb*B-2 product is an atypical receptor-like tyrosine kinase with constitutive activity in the absence of ligand. New Biologist 1990;2:992-1003.
10. Carter P, Presta L, Gorman CM, Ridgway JBB, Henner D, Wong WLT, et al. Humanization of an anti-p185^{HER2} antibody for human cancer therapy. Proc Natl Acad Sci USA 1992;89:4285-9.
11. Hudziak RM, Lewis GD, Winget M, Fendly BM, Shepard HM, Ullrich A. p185^{HER2} monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. Mol Cell Biol 1989;9:1165-72.
12. Lewis GD, Figari I, Fendly B, Wong WL, Carter P, Gorman C, et al. Differential responses of human tumor cell lines to anti-p185^{HER2} monoclonal antibodies. Cancer Immunol Immunother 1993;37:255-63.
13. Baselga J, Norton L, Albanell J, Kim Y-M, Mendelsohn J. Recombinant humanized anti-HER2 antibody (Herceptin™) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/*neu* overexpressing human breast cancer xenografts. Cancer Res 1998;58:2825-31.
14. a. Medical Laboratories – Particular requirements for quality and competence, ISO 15189:2003. b. Clinical Laboratory Improvement Amendments of 1988: Final Rule, 57CFR7163, February 28, 1992.
15. Sheehan DC, Hrapchak BB. Theory and practice of histotechnology. St. Louis: The C. V. Mosby Company; 1980.
16. Kiernan JA. Histological and histochemical methods: theory & practice. New York: Pergamon Press; 1981.
17. Lundgaard Hansen B, Winther H, Moller K. Excessive section drying of breast cancer tissue prior to deparaffinisation and antigen retrieval causes a loss in HER2 immunoreactivity, Immunocytochemistry 2008;6,119-22.
18. DakoCytomation California Inc., Data on file.
19. Key M. Education Guide: Immunohistochemical Staining Methods. Fifth Edition. Dako, Carpinteria, California; 2009.
20. Leong AS-Y, Milios J, Leong FJ. Epitope retrieval with microwaves. A comparison of citrate buffer and EDTA with three commercial retrieval solutions. Appl Immunohistochem 1996;4:201-7.
21. "Procedures for decontamination of plumbing systems containing copper and/or lead azides", Department of Health, Education, and Welfare, National Institute for Occupational Safety and Health, Rockville, MD, 1976.
22. "Decontamination of laboratory sink drains to remove azide salts." Center for Disease Control Manual Guide: Safety Management, No. CDC-22, Atlanta, GA, April 30, 1976.
23. Clinical and Laboratory Standards Institute (formerly NCCLS). Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline – Third Edition. CLSI document M29-A3 [ISBN 1-56238-567-4]. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2005.

24. Clinical and Laboratory Standards Institute (formerly NCCLS). Quality assurance for immunocytochemistry; Approved guideline. CLSI document MM4-A (1-56238-396-5) CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA; 1999.
25. Elias JM, Gown AM, Nakamura RM, Wilbur DC, Herman GE, Jaffe ES, et al. Special report: quality control in immunohistochemistry. Am J Clin Pathol 1989;92:836-43.
26. Nadji M, Morales AR. Immunoperoxidase: Part I. The technique and its pitfalls. Lab Med 1983;14:767-71.
27. Omata M, Liew C-T, Ashcavai M, Peters RL. Nonimmunologic binding of horseradish peroxidase to hepatitis B surface antigen: a possible source of error in immunohistochemistry. Am J Clin Path 1980;73:626-32.
28. Herman GE, Elfont EA. The taming of immunohistochemistry: the new era of quality control. Biotech & Histochem 1991; 66:194-9.
29. Press MF, Hung G, Godolphin W, Slamon DJ. Sensitivity of HER-2/*neu* antibodies in archival tissue samples: potential source of error in immunohistochemical studies of oncogene expression. Cancer Res 1994;54:2771-7.
30. Tubbs RR, Gephardt GN, Petras RE. Specimen processing and quality assurance. In: Atlas of immunohistology. Chicago: Amer Soc Clin Pathol Press; 1986:16-27.
31. Nadji M, Morales AR. Immunoperoxidase techniques. A practical approach to tumor diagnosis. Chicago: Amer Soc Clin Pathol Press; 1986.
32. Jørgensen JT. Targeted HER2 Treatment in Advanced Gastric Cancer. Oncology 2010; 78:26-33.
33. Tanner M, Hollmén M, Junttila TT, Kapanen AI, Tomola S, Soini Y, et al. Amplification of HER-2 in gastric carcinoma: association with Topoisomerase II alpha gene amplification, intestinal type, poor prognosis and sensitivity to trastuzumab. Ann Oncol 2005:16:273-8.
34. Matsui Y, Inomata M, Tojigamori M, Sonoda K, Shiraishi N, Kitano S. Suppression of tumor growth in human gastric cancer with HER2 overexpression by an anti-HER2 antibody in a murine model. Int J Oncol 2005; 27: 681-5.
35. Fujimoto-Ouchi K, Sekiguchi F, Yasuno H, Moriya Y, Mor K, Tanaka Y. Antitumor activity of trastuzumab in combination with chemotherapy in human gastric cancer xenograft models. Cancer Chemother Pharmacol 2007; 59: 795-805.
36. Kim SY, Kim HP, Kim YJ, Oh DY, Im S-A, Lee D, et al. Trastuzumab inhibits the growth of human gastric cancer cell lines with HER2 amplification synergistically with cisplatin. Int J Oncol 2008;32: 89-95.
37. Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-esophageal junction cancer (ToGA): a phase 3, open-label, randomized controlled trial. Lancet 2010; DOI:10.1016/S0140-6736(10)61121-x.
38. Bang Y, Chung J, Xu J, Lordick F, Sawaki A, Lipatov O, et al. Pathological features of advanced Gastric Cancer (GC): Relationship to human epidermal growth factor receptor 2 (HER2) positivity in the global screening programme of the ToGA trial. J Clin Oncol 2009; 27:15s (suppl; abstr 4556).
39. 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.

Explanation of symbols

REF	Catalogue number	2°C 8°C	Temperature limitation	LOT	Batch code	Xn	Harmful
IVD	In vitro diagnostic medical device		Fragile handle with care		Use by		
	Consult instructions for use	Σ	Contains sufficient for <n> tests		Manufacturer		

Produced by:

Dako Denmark A/S
Produktionsvej 42
DK-2600 Glostrup
Denmark/ Danemark/ Dänemark
Tel. +45 44 85 95 00
Fax +45 44 85 95 95

Distributed in the USA by:

Dako North America, Inc.
6392 Via Real
Carpinteria, California 93013, USA
Tel. 805/566-6655
Toll Free 800/235-5743
Ordering Information
Tel. 800/235-5763
Fax 805/566-6688
Technical ServicesTel. 800/424-0021