



**HercepTest™  
for Automated Link Platforms**

**Code SK001**

14th edition

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**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 breast and gastric cancer patients for whom Herceptin® (trastuzumab) treatment is being considered and for breast cancer patients for whom PERJETA™ (pertuzumab) or KADCYLA™ (ado-trastuzumab emtansine) treatment is being considered (see Herceptin®, PERJETA™ and KADCYLA™ package inserts).

**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  $\geq 2.0$ ). 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.

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

*HercepTest™, Herceptin®, PERJETA™ and KADCYLA™ (ado-trastuzumab emtansine) are trademarks owned by Genentech, Inc. and/or F. Hoffmann-La Roche Ltd.; HercepTest™ is subject to an exclusive trademark license to Agilent Technologies Singapore (International) Pte Ltd.*

For breast cancer application, please refer to pages 4-22.

For gastric cancer application, please refer to pages 23-42.

**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 p185HER2. 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).

Pertuzumab is a recombinant humanized monoclonal antibody that binds to sub-domain II of the extracellular part of the HER2 protein thereby blocking its ability to form heterodimers with other members of the HER family including HER1 (EGFR), HER3, and HER4 (14-16). PERJETA™ (pertuzumab) has shown to be effective and safe in treatment of breast cancer patients with HER2 protein overexpression. During clinical studies of pertuzumab, HER2 overexpression was demonstrated directly by IHC or indirectly evidenced through correlation of HER2 gene amplification to protein overexpression as demonstrated by FISH. However, in the randomized trial, data were available for a limited number of patients (8/808) for whom the FISH results were positive but the IHC results were negative (0, 1+) (17, 18).

Ado-trastuzumab emtansine is a novel antibody–drug conjugate specifically designed for the treatment of HER2-positive cancer. It is composed of the potent cytotoxic agent DM1 (a thiol-containing maytansinoid anti-microtubule agent) conjugated to trastuzumab via a linker molecule. Ado-trastuzumab emtansine binds to HER2 with an affinity similar to that of trastuzumab; such binding is required for its anti-tumor activity. It is hypothesized that after binding to HER2, ado-trastuzumab emtansine undergoes receptor-mediated internalization, followed by intracellular release of DM1 and subsequent cytotoxicity (19). A number of clinical studies have shown that ado-trastuzumab emtansine is effective and safe in treatment of HER2-positive breast cancer patients (20, 21, 22, 23).

### *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 immunocytochemical 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™ for Automated Link Platforms, Code SK001, is applicable for automated staining using Dako Automated Link platforms.

**Materials provided -  
Breast****Code SK001**

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 tissue section (22 mm x 22 mm) of each reagent except for Epitope Retrieval Solution. The kit provides materials sufficient for a maximum of 10 individual staining runs.

<i>Quantity</i>	<i>Description</i>
1 x 22 mL	<div style="border: 1px solid black; padding: 2px; width: fit-content;">HercepTest™ PEROXIDASE-BLOCKING REAGENT</div> <p><b>HercepTest™ Peroxidase-Blocking Reagent</b> 3% hydrogen peroxide containing 15 mmol/L sodium azide (NaN<sub>3</sub>).</p>
1 x 12 mL	<div style="border: 1px solid black; padding: 2px; width: fit-content;">HercepTest™ RABBIT ANTI-HUMAN HER2 PROTEIN</div> <p><b>HercepTest™ Rabbit Anti-Human HER2 Protein</b> Ready-to-use affinity-isolated antibody. Supplied in 0.05 mol/L Tris/HCl, 0.1 mol/L NaCl, 15 mmol/L NaN<sub>3</sub>, 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.</p>
1 x 22 mL	<div style="border: 1px solid black; padding: 2px; width: fit-content;">HercepTest™ VISUALIZATION REAGENT</div> <p><b>HercepTest™ Visualization Reagent</b> 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.</p>
1 x 10 mL	<div style="border: 1px solid black; padding: 2px; width: fit-content;">HercepTest™ NEGATIVE CONTROL REAGENT</div> <p><b>HercepTest™ Negative Control Reagent</b> 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<sub>3</sub>, pH 7.2, containing stabilizing protein.</p>
2 x 22 mL	<div style="border: 1px solid black; padding: 2px; width: fit-content;">HercepTest™ DAB SUBSTRATE BUFFER</div> <p><b>HercepTest™ DAB Substrate Buffer</b> Substrate buffer solution, pH 7.5, containing &lt; 0.1% hydrogen peroxide, stabilizers, enhancers, and an antimicrobial agent.</p>
1 x 1 mL	<div style="border: 1px solid black; padding: 2px; width: fit-content;">HercepTest™ DAB CHROMOGEN</div> <p><b>HercepTest™ DAB Chromogen</b> 5% 3,3'-diaminobenzidine tetrahydrochloride chromogen solution.</p>
3 x 500 mL	<div style="border: 1px solid black; padding: 2px; width: fit-content;">EPI TOPE RETRIEVAL SOLUTION (CONTAINING DETERGENT) (10x)</div> <p><b>HercepTest™ Epitope Retrieval Solution (Containing Detergent 10x)</b> 0.1 mol/L citrate buffer with a detergent.</p>
2 x 5 slides	<div style="border: 1px solid black; padding: 2px; width: fit-content;">HercepTest™ CONTROL SLIDES</div> <p><b>HercepTest™ Control Slides</b> 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.</p>
10 x bottles	<div style="border: 1px solid black; padding: 2px; width: fit-content;">USER-FILLABLE REAGENT BOTTLE 12 mL CAPACITY</div> <p><b>User-Fillable Reagent Bottle, 12 mL Capacity</b> Each bottle can contain 12 mL reagent. The bottles should be used for mixing and containing Substrate-Chromogen Solution (see also Reagent Preparation section).</p>

**NOTE:** All reagents are formulated specifically for use with this test. For the test to perform as specified, no substitutions should be made.

**Materials required,  
but not supplied -  
Breast**

**Dako Wash Buffer, Code S3006**

Counterstain: Hematoxylin, such as Hematoxylin for Automated Link Platforms, (Code SK308)

Coverslips

Distilled or deionized 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 Dako 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)

Xylene, toluene, or xylene substitutes

SK001 has been tailored for use with the Dako Automated Link platforms. Please refer to the User Guide for your Dako Automated Link Platform(s) for further information.

## Precautions - Breast

1. For in vitro diagnostic use.
2. For professional users.
3. This product contains sodium azide (NaN<sub>3</sub>), 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 (31).
4. HercepTest™ Peroxidase-Blocking Reagent, contains 3% hydrogen peroxide. Safety data sheet available for professional users on request.
5. HercepTest™ Rabbit Anti-Human HER2 Protein, HercepTest™ Visualization Reagent and HercepTest™ Negative Control Reagent contain material of animal origin.
6. As with any product derived from biological sources, proper handling procedures should be used.
7. 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 (32). 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.
8. Minimize microbial contamination of reagents or increase in nonspecific staining may occur.
9. 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 (27).
10. Reagents have been optimally diluted. Further dilution may result in loss of antigen staining.
11. All reagents are formulated specifically for use with this test. In order for the test to perform as specified, no substitutions should be made.
12. The HercepTest™ Visualization Reagent and HercepTest™ 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.
13. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.
14. Unused solution should be disposed of according to local, State and Federal regulations.
15. Paraffin residuals may lead to false negative results.
16. Use of reagent volumes other than recommended may result in loss of visible HER2 immunoreactivity. Tissue sections larger than 22 mm x 22 mm will require 2-3x 200 µL of reagent applied in 2-3 automated application areas.
17. HercepTest™ DAB Chromogen, contains ≥75-≤90% propane-1,2-diol, ≤10% 3,3'-diaminobenzidine tetrahydrochloride (biphenyl-3,3',4,4'- tetrayltetraammonium tetrachloride) and is labeled:



### Danger

- |                |                                                                                                                                  |
|----------------|----------------------------------------------------------------------------------------------------------------------------------|
| H319           | Causes serious eye irritation.                                                                                                   |
| H350           | May cause cancer.                                                                                                                |
| H341           | Suspected of causing genetic defects.                                                                                            |
| P201           | Obtain special instructions before use.                                                                                          |
| P202           | Do not handle until all safety precautions have been read and understood.                                                        |
| P280           | Wear protective gloves. Wear eye or face protection. Wear protective clothing.                                                   |
|                |                                                                                                                                  |
| P264           | Wash hands thoroughly after handling.                                                                                            |
| P308 + P313    | IF exposed or concerned: Get medical attention.                                                                                  |
| P305+P351+P338 | IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. |
| P337+P313      | If eye irritation persists: Get medical attention.                                                                               |
| P405           | Store locked up.                                                                                                                 |
| P501           | Dispose of contents and container in accordance with all local, regional, national and international regulations.                |
18. HercepTest™ DAB Substrate Buffer contains <1% imidazole and is labeled:



### Danger

- |             |                                                                                                                   |
|-------------|-------------------------------------------------------------------------------------------------------------------|
| H360        | May damage the unborn child.                                                                                      |
| P201        | Obtain special instructions before use.                                                                           |
| P202        | Do not handle until all safety precautions have been read and understood.                                         |
| P280        | Wear protective gloves. Wear eye or face protection. Wear protective clothing.                                    |
| P308 + P313 | IF exposed or concerned: Get medical attention.                                                                   |
| P405        | Store locked up.                                                                                                  |
| P501        | Dispose of contents and container in accordance with all local, regional, national and international regulations. |

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. Please refer to the Safety Data Sheet (SDS) for additional information.

## Storage - Breast

Store at 2–8 °C when not in use on Dako Automated Link Platforms. Store HercepTest™ Peroxidase-Blocking Reagent, HercepTest™ Visualization Reagent, HercepTest™ DAB Substrate Buffer and HercepTest™ DAB Chromogen in the dark 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 (24a,24b). 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 Support.

## 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 (25).

### *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) (25, 26). 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" (26).

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 (27).

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) (28). 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 Rabbit Anti-Human HER2 Protein and 1 for incubation with Negative Control Reagent), and 2 slides for back-up. To preserve antigenicity, tissue sections mounted on slides (SuperFrost Plus, poly-L-lysine or silanized slides) should be stained within 4–6 weeks of sectioning when held at room temperature (20–25 °C) (28).

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

Consult Dako's "Education Guide: Immunohistochemical Staining Methods" (29) and references 25 and 26 for further details on specimen preparation.

### *Treatment of tissues prior to staining*

A specific epitope retrieval method in 0.01 mol/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 0.01 mol/L citrate buffer (30) in a calibrated water bath or Dako PT Link (Code PT101 or PT200) at the required temperature (95–99 °C). Laboratories located at higher elevations should determine the best method of maintaining the required temperature. Other methods of epitope retrieval have been tested and do not give reproducible results. Deviation from the described procedure may affect results.

## Reagent preparation - Breast

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

### *Epitope Retrieval Solution*

Dilute a sufficient quantity of HercepTest™ Epitope Retrieval Solution 1:10 using distilled or deionized water for the staining procedure that is planned. Unused diluted solution may be used for one week at room temperature or stored at 2–8 °C for one month. Discard diluted solution if cloudy in appearance. Refer to the User Guide for your Dako Automated Link Platform(s) for further information.

### *Wash Buffer*

Dilute a sufficient quantity of Dako Wash Buffer (Code S3006) 1:10 using distilled or deionized water. Unused diluted buffer may be used for one week at room temperature or stored at 2–8 °C for one month. Discard buffer if cloudy in appearance. Refer to the User Guide for your Dako Automated Link Platform(s) for further information.



## *Substrate-Chromogen Solution (DAB)*

Prepare Substrate-Chromogen Solution in the user-fillable bottles by adding 25 µL DAB Chromogen per 1 mL of DAB Substrate Buffer 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.

The user-fillable bottle containing mixed Substrate-Chromogen Solution needs to be defined in the Dako Automated Link Platform before use. Refer to instrument User Guide for your Dako Automated Link Platform(s) for further information.

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

## *Counterstain*

The colored end-product of the DAB staining reaction is alcohol and water insoluble. Hematoxylin for Automated Link Platforms (Code SK308) may be used as a counterstain. The required steps and incubation times are preprogrammed in the Dako Link software. No reagent preparation is required.

## *Mounting Medium*

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

## **Staining procedure - Breast**

### *Procedural notes*

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

Do not allow tissue sections to dry at any time during the staining procedure. Dried tissue sections may display increased non-specific staining.

All of the required steps and incubation times for staining are preprogrammed in the Dako Link software. Please refer to the User Guide for your Dako Automated Link Platform(s) for further information on programming protocols and loading slides and reagents. 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 the section staining protocol under Epitope retrieval.

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

**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.

### *Staining protocol*

All of the required steps and incubation times for staining are preprogrammed in the Dako Automated Link Platforms software. After deparaffinization, rehydration and epitope retrieval, the instrument will process the slides as described below.

**Step 1: Epitope retrieval**

Fill the Dako PT Module tanks or staining jar e.g. Coplin jar, with Epitope Retrieval Solution (see reagent preparation).

For the Dako PT Link (Code PT101 or PT200): Pre-heat the diluted Epitope Retrieval Solution in the Dako PT Link tank to 85 °C. Upon heating the Epitope Retrieval Solution becomes cloudy in appearance. Place the room temperature deparaffinized sections in Autostainer Racks and immerse the slides in the preheated Epitope Retrieval Solution. Let the Dako PT Link warm up to 97 °C and incubate for 40 (±1) minutes at 97 °C. Leave the sections to cool in the Dako PT Link until the temperature reaches 85 °C. Remove the PT Link tanks with the sections from the Dako PT Link. Leave the tanks at the table for 10 minutes with the lid off for further cooling. Rinse sections with Wash Buffer. See the Dako PT Link User Guide for more information.

For Coplin jars: Place staining jars containing diluted 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 Epitope retrieval solution back to 95–99 °C.** Upon heating the Epitope Retrieval Solution becomes cloudy in appearance. Incubate for 40 (±1) minutes at 95–99 °C. Remove the entire jar with slides from the water bath. Allow 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.

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: Staining procedure**

After epitope retrieval, the slides placed in instrument racks are placed on the Dako Automated Link Platforms. The instrument will perform the staining process by applying the appropriate reagent, monitoring the incubation time and rinsing the slides between reagents. The reagent incubation times are preprogrammed in the Dako Link software.

**Step 3: Counterstain**

Slides may be counterstained with Hematoxylin for Automated Link Platforms (Code SK308). There are 2 staining protocols provided in the Dako software. One includes a hematoxylin counterstain and the other does not. The hematoxylin incubation time is preprogrammed in the protocol that includes a counterstain. See the User Guide for your Dako Automated Link Platform(s) for further information on programming protocols.

**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 Dako 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 (33) and reference 34 for additional information.

**Table 1: The purpose of daily quality control.**

<i>Tissue: Fixed and Processed Like Patient Sample</i>	<i>Specific Antibody and Detection System</i>	<i>Non-Specific Antibody* or Buffer Plus Same Detection System as Used with Specific Antibody</i>
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.

See the User Guide for your Dako Automated Link Platform(s) for further information on control slide programming.

### *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 a staining system in a diagnostic procedure, the user should verify the assay's performance by testing it on a series of in-house tissues with known IHC 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 (33). 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.

**Staining interpretation - 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.**

<i>Staining pattern</i>	<i>Score (Report to treating physician)</i>	<i>HER2 Protein Overexpression Assessment (Report to treating physician)</i>
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.**

<i>Slide Reading Order</i>	<i>Rationale</i>
1. Control Slide containing the three cell lines  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 <b>ABSENCE</b> 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 labelling 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 (35). 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 labelling 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.

1. Evaluate the Control Cell Lines to validate the assay performance.
2. Evaluate the Positive and Negative Control Slides.
3. A hematoxylin and eosin (H&E) 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.
4. Evaluate the sections stained for HER2 protein overexpression at low power first. The majority of positive cases will be obvious at low power magnification.
5. Well-preserved and well-stained areas of the specimen should be used to make a determination of the percent positive tumor cells.
6. 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+.
7. To verify membrane staining, use 20–40x objective magnification.
8. 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.
9. In a majority of the 3+ cases, at least 80% of the tumor cells are stained and the membrane staining is intense.
10. 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.
11. 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.
12. 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+.
13. If less than 10% of the tumor cells have complete or incomplete circumferential membrane staining, the score is 0.

## General limitations - Breast

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 (36).
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 (37). Contact Dako Technical Support with documented unexpected reactions.
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) (37).
8. The staining procedure will be performed at the preprogrammed temperature (20–25 °C).

## Product specific limitations - Breast

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) (38).
3. Do not replace kit reagents, with reagents carrying other lot numbers or with reagents from other manufacturers.
4. False results could be obtained from evaluation of cytoplasmic staining. Consider only the intensity of cell membrane staining when interpreting results.
5. 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.
6. 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.
7. Use of HercepTest™ on specimens fixed in fixatives other than neutral buffered formalin or Bouin’s fixative has not been validated.
8. 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		Total
		Positive	Negative	
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.**

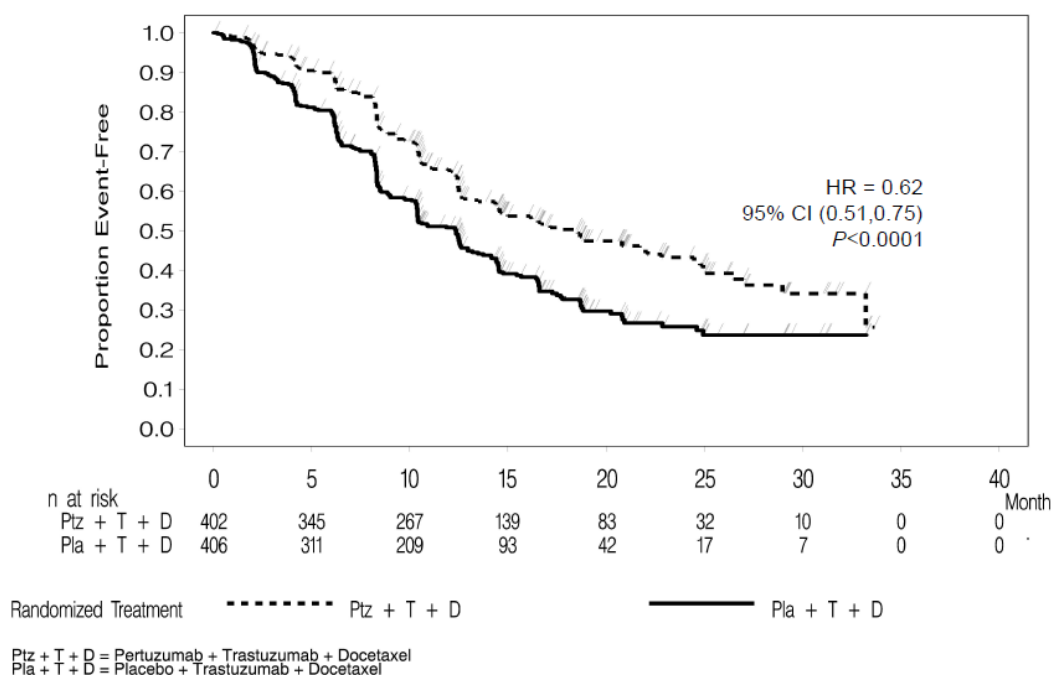
		Clinical Trial Assay			Total
		3+	2+	0 - 1+	
HercepTest	3+	107	36	6	149
	2+	16	57	53	126
	0 - 1+	8	50	215	273
Total		131	143	274	548

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.

The clinical safety and efficacy of PERJETA™ (pertuzumab) has been demonstrated in the randomized study (18). The study was designed as a randomized, double-blinded, placebo-controlled phase III trial in patients with HER2-positive metastatic or locally recurrent, unresectable breast cancer who had not received previous treatment or whose disease has relapsed after adjuvant therapy. After inclusion in the study the patients were randomized 1:1 to receive placebo plus Herceptin and docetaxel or PERJETA plus Herceptin and docetaxel. In the randomized trial, HER2 positivity was defined as being either IHC positive (3+) or FISH positive (HER2/CEN-17  $\geq$  2.0) tested with HercepTest™, Dako or HER2 FISH pharmDx™ Kit, Dako, respectively. Cases that were FISH negative but IHC positive (3+) were enrolled. Cases that were IHC negative (0, 1+) that were FISH positive (HER/CEN-17  $\geq$  2.0) were enrolled. However limited data (8/808) were available for cases that were positive only by FISH (no direct evidence of protein overexpression) and as such, caution should be exercised when interpreting these results.



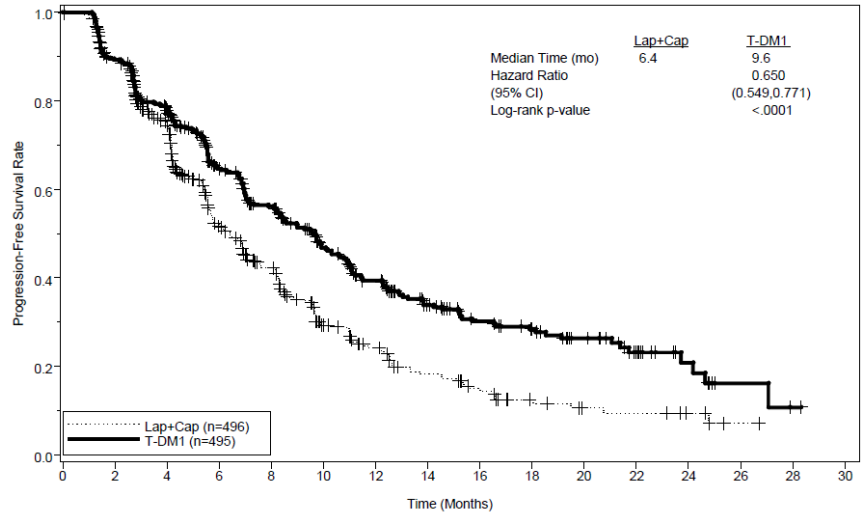
In the randomized study a total of 808 patients were enrolled, of whom 406 patients were randomized to the placebo treated group and 402 patients to the PERJETA™ treated group. The primary efficacy endpoint in the study was progression-free survival (PFS) based on tumor assessments by an independent review facility (IRF). At the time of the primary analysis of PFS, a total of 242 patients (59.6%) in the placebo treated group and 191 patients (47.5%) in the PERJETA™ treated group had IRF confirmed progressive disease or had died within 18 weeks of their last tumor assessment. The randomized study demonstrated a statistically significant improvement in IRF-assessed PFS (hazard ratio [HR] = 0.62, 95% CI = 0.51; 0.75,  $p < 0.0001$ ) in the PERJETA treated group compared with the placebo treated group, and an increase in median PFS of 6.1 months (median PFS of 12.4 months in the placebo treated group vs. 18.5 months in the PERJETA treated group). Figure 1 shows the Kaplan-Meier Curve of IRF-assessed PFS.



**Figure 1.** Kaplan-Meier Curve of IRF-assessed Progression Free Survival.

The clinical safety and efficacy of KADCYLA™ (ado-trastuzumab emtansine) has been demonstrated in a phase III study (EMILIA) (21). The study was designed as a randomized, multicenter, international, two-arm, open-label clinical trial in patients with centrally confirmed HER2-positive, unresectable locally advanced or metastatic breast cancer who have previously received a taxane and trastuzumab and have progressive disease at study entry. After inclusion in the study the patients were randomized 1:1 to receive lapatinib and capecitabine or ado-trastuzumab emtansine. In the study, HER2 positivity was defined as being IHC positive (3+) and/or FISH positive. The majority of patients (881/991) had a test result available with both Dako HercepTest™ and Dako HER2 FISH pharmDx™ Kit. Cases that were IHC negative (0, 1+) that were FISH positive (HER2/CEN-17  $\geq 2.0$ ) were enrolled. However limited data were available for cases that were positive by FISH but negative by IHC (11/991) (no direct evidence of protein overexpression) and as such, caution should be exercised when interpreting these results.

In the phase III study a total of 991 patients were enrolled, of whom 496 patients were randomized to the lapatinib and capecitabine group and 495 patients to the ado-trastuzumab emtansine group. The co-primary efficacy endpoints in the study were progression-free survival (PFS) based on tumor assessments by an independent review committee (IRC) and overall survival (OS). As of the clinical data cutoff of 14 January 2012, 182 patients (37.1%) in the ado-trastuzumab emtansine arm and 125 patients (25.6%) in the lapatinib plus capecitabine arm were still alive and on treatment. The randomized phase III study showed that treatment with ado-trastuzumab emtansine was associated with a 35% reduction in the risk of disease progression or death (hazard ratio of (HR) = 0.650 (95% CI = 0.549, 0.771;  $p < 0.0001$ )) compared to lapatinib and capecitabine. The Kaplan-Meier curve showed a clear separation at approximately 4 months that was maintained from that point onward (Figure 2). A 50% relative increase in median PFS (by IRC) was observed in patients treated with ado-trastuzumab emtansine compared with patients treated with lapatinib plus capecitabine (9.6 months vs. 6.4 months).

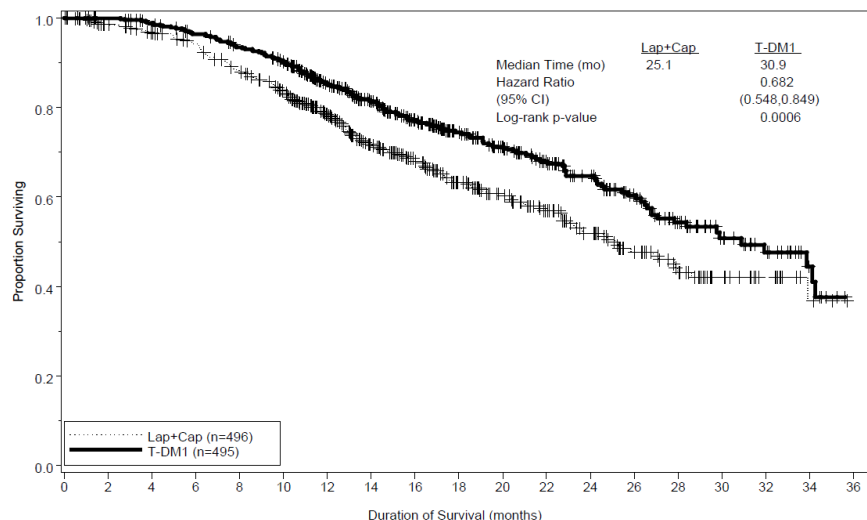


Number at Risk:	0	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
Lap+Cap	496	404	310	176	129	73	53	35	25	14	9	8	5	1	0	0
T-DM1	495	419	341	236	183	130	101	72	54	44	30	18	9	3	1	0

T-DM1: ado-trastuzumab emtansine; Lap: lapatinib; Cap: capecitabine; IRC: independent review committee.  
Hazard ratio is estimated based on a stratified Cox model, p-value is estimated based on a stratified log-rank test.

**Figure 2.** Kaplan-Meier Curve of IRC-assessed Progression Free Survival.

At the time of PFS analysis, 223 patients had died. More deaths occurred in the lapatinib plus capecitabine arm (26%) compared with the KADCYLA arm (19%), however the results of this interim OS analysis did not meet the pre-specified stopping boundary for statistical significance. A second interim OS analysis was planned for when at least 50% of the target 632 events for final analysis had occurred. At the time of the second interim analysis, 331 events (representing 52.4% of targeted events) had occurred. The co-primary endpoint of OS was met; OS was significantly improved in patients receiving KADCYLA (HR = 0.682, 95% CI: 0.548, 0.849, p = 0.0006; See Figure 3). This result crossed the pre-specified efficacy stopping boundary (HR = 0.727 or p = 0.0037) and represents the confirmatory analysis for OS. The median duration of survival was 30.9 months in the KADCYLA arm vs. 25.1 months in the lapatinib plus capecitabine arm.



Number at Risk:	0	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
Lap+Cap	496	471	453	435	403	368	297	240	204	159	133	110	86	63	45	27	17	7	4
T-DM1	495	485	474	457	439	418	349	293	242	197	164	136	111	86	62	38	28	13	5

T-DM1: ado-trastuzumab emtansine; Lap: lapatinib; Cap: capecitabine.  
Hazard ratio is estimated based on a stratified Cox model, p-value is estimated based on a stratified log-rank test.

**Figure 3.** Kaplan-Meier Curve of Overall Survival.

*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 (38). 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, ductal epithelium (2 of 3 tissues, 1+ staining intensity, >10% of cells, incomplete membrane)
Cervix uteri (3)	None
Colon (3)	None
Esophagus (3)	None
Heart (3)	None
Kidney (3)	None
Liver (3)	None
Lung (3)	None
Mesothelial cells (3)	None
Ovary (3)	None
Pancreas (3)	None
Parathyroid (3)	Chief cells (Oxyphil cells 2 of 3 tissues, 1+ staining intensity, >50% of cells, membrane and cytoplasm)
Peripheral nerve (3)	None
Pituitary (3)	None
Prostate (3)	None
Salivary gland (3)	None
Skeletal muscle (3)	None
Skin (3)	Basal layer epithelium (2 of 3 tissues, 1-2+ staining intensity, >10% of cells, membrane and cytoplasm)
Small intestine (3)	None
Spleen (3)	None
Stomach (3)	Chief-cells (1 of 3 tissues, 1-2+ staining intensity, >10 % of cells, cytoplasm)
Testis (3)	None
Thymus (3)	None
Thyroid (3)	None
Tonsil (3)	None
Uterus (3)	Glandular epithelial cells (2 of 3 tissues, 0-1+ staining intensity, >50% of cells, cytoplasm)

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 (29) for remedial action, or contact Dako Technical Support Department to report unusual staining.

<i>Problem</i>	<i>Probable Cause</i>	<i>Suggested Action</i>
1. No staining of slides.	1a. Programming error. Reagents not used in proper order. 1b. Sodium azide in Wash Solution. 1c. Excessive heating of mounted tissue sections prior to deparaffinization and heat-induced antigen retrieval may lead to loss of visible HER2 immunoreactivity.	1a. Check slide log to verify that correct program was selected. 1b. Use only Dako Wash Buffer, Code S3006. 1c. 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 (27).
2. Weak staining of slides.	2a. Inadequate epitope retrieval. 2b. Inadequate reagent incubation times. 2c. Inappropriate fixation method 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. 2e. Insufficient reagent volume applied.	2a. Review the slide log to confirm that epitope retrieval was performed correctly. 2b. Review the slide log to confirm that reagent incubation times are correct. 2c. Ensure that patient tissue is not over-fixed or that an alternative fixative was not used. 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 (27). 2e. Check size of tissue section (22 mm x 22 mm) and reagent volume applied.
3. Excessive background staining of slides.	3a. Paraffin incompletely removed. 3b. Starch additives used in mounting sections to slides. 3c. Slides not thoroughly rinsed. 3d. Sections dried during staining procedure. 3e. Inappropriate fixation method used. 3f. Non-specific binding of reagents to tissue.	3a. Use fresh deparaffinization solutions. 3b. Avoid using starch additives for adhering sections to glass slides. Many additives are immunoreactive. 3c. Review the slide log to confirm that slides were rinsed properly. 3d. Verify that the appropriate volume of reagent is applied to slides. 3e. Ensure that approved fixative was used. Alternative fixative may cause excessive back-ground staining. 3f. 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.

<i>Problem</i>	<i>Probable Cause</i>	<i>Suggested Action</i>
5. Excessively strong specific staining.	5a. Inappropriate fixation method used. 5b. Reagent incubation times too long. 5c. Inappropriate wash solution used.	5a. Ensure that only approved fixatives and fixation methods are used. 5b. Review the slide log to confirm that reagent incubation times are correct. 5c. Use only Dako Wash Buffer, Code S3006.
6. Weak staining of the 1+ Control Slide Cell Line.	6a. Incorrect epitope retrieval protocol followed. 6b. Lack of reaction with Substrate-Chromogen Solution (DAB). 6c. Degradation of Control Slide.	6a. Review the slide log to confirm that epitope retrieval was performed correctly. 6b. Review the run log to confirm that the chromogen was prepared correctly. Review the slide log to confirm that the chromogen incubation time is correct. 6c. Check kit expiration date and kit storage conditions on outside of package.
7. Epitope Retrieval Solution is cloudy in appearance when heated.	7. When heated the solution turns cloudy in appearance.	7. This is normal and does not influence the staining.
8. Epitope Retrieval Solution is cloudy in appearance when stored (prior to heating).	8. The solution has been incorrectly stored or the expiration date of the solution has passed.	8. Check the expiration date and kit storage conditions on outside of package. Discard the Epitope Retrieval Solution.

**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 Technical Support for further assistance. Additional information on staining techniques and specimen preparation can be found in Dako previously referenced Handbook (29) (available from Dako), Atlas of Immunohistology (39) and Immunoperoxidase Techniques. A Practical Approach to Tumor Diagnosis (40).

## 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<sup>HER2</sup>. 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 (41). HER2 positivity can be detected in approximately 20% of the patients by either IHC or FISH (41). 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 (41-45).

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*  $\geq$  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 (46, 47). 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 (42-45).

### *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*  $\geq$  2.0). The results from this study showed that ~22% of the patients with advanced gastric or gastroesophageal junction adenocarcinoma were HER2 positive (47).

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 immunocytochemical 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™ for Automated Link Platforms, Code SK001, is applicable for automated staining using Dako Automated Link platforms.

## Materials provided - Gastric

### Code SK001

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 tissue section (22 mm x 22 mm) of each reagent except for Epitope Retrieval Solution. The kit provides materials sufficient for a maximum of 10 individual staining runs.

Quantity	Description
1 x 22 mL	<div style="border: 1px solid black; padding: 2px; width: fit-content;">HercepTest™ PEROXIDASE-BLOCKING REAGENT</div> <p><b>HercepTest™ Peroxidase-Blocking Reagent</b> 3% hydrogen peroxide containing 15 mmol/L sodium azide (NaN<sub>3</sub>).</p>
1 x 12 mL	<div style="border: 1px solid black; padding: 2px; width: fit-content;">HercepTest™ RABBIT ANTI-HUMAN HER2 PROTEIN</div> <p><b>HercepTest™ Rabbit Anti-Human HER2 Protein</b> Ready-to-use affinity-isolated antibody. Supplied in 0.05 mol/L Tris/HCl, 0.1 mol/L NaCl, 15 mmol/L NaN<sub>3</sub>, 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.</p>
1 x 22 mL	<div style="border: 1px solid black; padding: 2px; width: fit-content;">HercepTest™ VISUALIZATION REAGENT</div> <p><b>HercepTest™ Visualization Reagent</b> 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.</p>
1 x 10 mL	<div style="border: 1px solid black; padding: 2px; width: fit-content;">HercepTest™ NEGATIVE CONTROL REAGENT</div> <p><b>HercepTest™ Negative Control Reagent</b> 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<sub>3</sub>, pH 7.2, containing stabilizing protein.</p>
2 x 22 mL	<div style="border: 1px solid black; padding: 2px; width: fit-content;">HercepTest™ DAB SUBSTRATE BUFFER</div> <p><b>HercepTest™ DAB Substrate Buffer</b> Substrate buffer solution, pH 7.5, containing &lt; 0.1% hydrogen peroxide, stabilizers, enhancers, and an antimicrobial agent.</p>
1 x 1 mL	<div style="border: 1px solid black; padding: 2px; width: fit-content;">HercepTest™ DAB CHROMOGEN</div> <p><b>HercepTest™ DAB Chromogen</b> 5% 3,3'-diaminobenzidine tetrahydrochloride chromogen solution.</p>
3 x 500 mL	<div style="border: 1px solid black; padding: 2px; width: fit-content;">EPI TOPE RETRIEVAL SOLUTION (CONTAINING DETERGENT) (10x)</div> <p><b>HercepTest™ Epitope Retrieval Solution (Containing Detergent 10x)</b> 0.1 mol/L citrate buffer with a detergent.</p>
2 x 5 slides	<div style="border: 1px solid black; padding: 2px; width: fit-content;">HercepTest™ CONTROL SLIDES</div> <p><b>HercepTest™ Control Slides</b> 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.</p>
10 x bottles	<div style="border: 1px solid black; padding: 2px; width: fit-content;">USER-FILLABLE REAGENT BOTTLE 12 mL CAPACITY</div> <p><b>User-Fillable Reagent Bottle, 12 mL Capacity</b> Each bottle can contain 12 mL reagent. The bottles should be used for mixing and containing Substrate-Chromogen Solution (see also Reagent Preparation section).</p>

**NOTE:** All reagents are formulated specifically for use with this test. For the test to perform as specified, no substitutions should be made.



**Materials required,  
but not supplied -  
Gastric**

**Dako Wash Buffer, Code S3006**

Counterstain: Hematoxylin, such as Hematoxylin for Automated Link Platforms, (Code SK308)

Coverslips

Distilled or deionized 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 Dako 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)

Xylene, toluene, or xylene substitutes

SK001 has been tailored for use with the Dako Automated Link platforms. Please refer to the User Guide for your Dako Automated Link Platform(s) for further information.

## Precautions - Gastric

1. For in vitro diagnostic use.
2. For professional users.
3. This product contains sodium azide (NaN<sub>3</sub>), 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 (31).
4. HercepTest™ Peroxidase-Blocking Reagent, contains 3% hydrogen peroxide. Safety data sheet available for professional users on request.
5. HercepTest™ Rabbit Anti-Human HER2 Protein, HercepTest™ Visualization Reagent and HercepTest™ Negative Control Reagent contain material of animal origin.
6. As with any product derived from biological sources, proper handling procedures should be used.
7. 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 (32). 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.
8. Minimize microbial contamination of reagents or increase in nonspecific staining may occur.
9. 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 (27).
10. Reagents have been optimally diluted. Further dilution may result in loss of antigen staining.
11. All reagents are formulated specifically for use with this test. In order for the test to perform as specified, no substitutions should be made.
12. The HercepTest™ Visualization Reagent and HercepTest™ 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.
13. 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.
14. 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.
15. 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.
16. It is recommended to test more than one tissue block of gastric cancer resections when the specimen exhibits a high level of heterogeneity (50)
17. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.
18. Unused solution should be disposed of according to local, State and Federal regulations.
19. Paraffin residuals may lead to false negative results.
20. Use of reagent volumes other than recommended may result in loss of HER2 immunoreactivity. Tissue sections larger than 22 mm x 22 mm will require 2-3x 200 µL of reagent applied in 2-3 automated application areas.
21. HercepTest™ DAB Chromogen, contains ≥75-≤90% propane-1,2-diol, ≤10% 3,3'-diaminobenzidine tetrahydrochloride (biphenyl-3,3',4,4'- tetrayltetraammonium tetrachloride) and is labeled:



### Danger

H350	May cause cancer.
H319	Causes serious eye irritation.
H341	Suspected of causing genetic defects.
P201	Obtain special instructions before use.
P202	Do not handle until all safety precautions have been read and understood.
P280	Wear protective gloves. Wear eye or face protection. Wear protective clothing.
P264	Wash hands thoroughly after handling.
P308 + P313	IF exposed or concerned: Get medical attention.
P305+P351+P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337+P313	If eye irritation persists: Get medical attention.
P405	Store locked up.
P501	Dispose of contents and container in accordance with all local, regional, national and international regulations.

22. HercepTest™ DAB Substrate Buffer contains <1% imidazole and is labeled:



### Danger

H360	May damage the unborn child.
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P201	Obtain special instructions before use.
P202	Do not handle until all safety precautions have been read and understood.
P280	Wear protective gloves. Wear eye or face protection. Wear protective clothing.
P308 + P313	IF exposed or concerned: Get medical attention.
P405	Store locked up.
P501	Dispose of contents and container in accordance with all local, regional, national and international regulations.

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. Please refer to the Safety Data Sheet (SDS) for additional information.

## Storage - Gastric

Store at 2–8 °C when not in use on Dako Automated Link Platforms. Store HercepTest™ Peroxidase-Blocking Reagent, HercepTest™ Visualization Reagent, HercepTest™ DAB Substrate Buffer and HercepTest™ DAB Chromogen in the dark 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 (24a,24b). 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 Support.

## 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 (25). 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 (46)). 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) (25,26). 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" (26).

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 (27).

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) (28). 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 Rabbit Anti-Human HER2 Protein and 1 for incubation with Negative Control Reagent), and 2 slides for back-up. To preserve antigenicity, tissue sections mounted on slides (SuperFrost Plus, poly-L-lysine or silanized slides) should be stained within 4–6 weeks of sectioning when held at room temperature (20–25 °C) (28).

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

Consult Dako's "Education Guide: Immunohistochemical Staining Methods" (29) and references 25 and 26 for further details on specimen preparation.

### *Treatment of tissues prior to staining*

A specific epitope retrieval method in 0.01 mol/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 0.01 mol/L citrate buffer (30) in a calibrated water bath or Dako PT Link (Code PT101 or PT200) at the required temperature (95–99 °C). Laboratories located at higher elevations should determine the best method of maintaining the required temperature. Other methods of epitope retrieval have been tested and do not give reproducible results. Deviation from the described procedure may affect results.

## Reagent preparation - Gastric

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

### *Epitope Retrieval Solution*

Dilute a sufficient quantity of HercepTest™ Epitope Retrieval Solution 1:10 using distilled or deionized water for the staining procedure that is planned. Unused diluted solution may be used for one week at room temperature or stored at 2–8 °C for one month. Discard diluted solution if cloudy in appearance. Refer to the User Guide for your Dako Automated Link Platform(s) for further information.

### *Wash Buffer*

Dilute a sufficient quantity of Dako Wash Buffer (Code S3006) 1:10 using distilled or deionized water. Unused diluted buffer may be used for one week at room temperature or stored at 2–8 °C for one month. Discard buffer if cloudy in appearance. Refer to the User Guide for your Dako Automated Link Platform(s) for further information.

## *Substrate-Chromogen Solution (DAB)*

Prepare Substrate-Chromogen Solution in the user-fillable bottles by adding 25  $\mu$ L DAB Chromogen per 1 mL of DAB Substrate Buffer 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.

The user-fillable bottle containing mixed Substrate-Chromogen Solution needs to be defined in the Dako Automated Link Platform before use. Refer to instrument User Guide for your Dako Automated Link Platform(s) for further information.

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

## *Counterstain*

The colored end-product of the DAB staining reaction is alcohol and water insoluble. Hematoxylin for Automated Link Platforms (Code SK308) may be used as a counterstain. The required steps and incubation times are preprogrammed in the Dako Link software. No reagent preparation is required.

## *Mounting Medium*

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

## Staining procedure - Gastric

### *Procedural notes*

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

Do not allow tissue sections to dry at any time during the staining procedure. Dried tissue sections may display increased non-specific staining.

All of the required steps and incubation times for staining are preprogrammed in the Dako Link software. Please refer to the User Guide for your Dako Automated Link Platform(s) for further information on programming protocols and loading slides and reagents. 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 ( $\pm$ 1) minutes. Change baths and repeat once.
2. Tap off excess liquid and place slides in absolute ethanol for 3 ( $\pm$ 1) minutes. Change baths and repeat once.
3. Tap off excess liquid and place slides in 95% ethanol for 3 ( $\pm$ 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 the section staining protocol under Epitope retrieval.

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

**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.

### *Staining protocol*

All of the required steps and incubation times for staining are preprogrammed in the Dako Automated Link Platforms software. After deparaffinization, rehydration and epitope retrieval, the instrument will process the slides as described below.

**Step 1: Epitope retrieval**

Fill the Dako PT Module tanks or staining jar e.g. Coplin jar, with Epitope Retrieval Solution (see reagent preparation).

For the Dako PT Link (Code PT101 or PT200): Pre-heat the diluted Epitope Retrieval Solution in the Dako PT Link tank to 85 °C. Upon heating the Epitope Retrieval Solution becomes cloudy in appearance. Place the room temperature deparaffinized sections in Autostainer Racks and immerse the slides in the preheated Epitope Retrieval Solution. Let the Dako PT Link warm up to 97 °C and incubate for 40 (±1) minutes at 97 °C. Leave the sections to cool in the Dako PT Link until the temperature reaches 85 °C. Remove the PT Link tanks with the sections from the Dako PT Link. Leave the tanks at the table for 10 minutes with the lid off for further cooling. Rinse sections with Wash Buffer. See the Dako PT Link User Guide for more information.

For Coplin jars: Place staining jars containing diluted Epitope Retrieval Solution in water bath. Heat water bath and the Epitope Retrieval Solution to 95–99 °C. Upon heating the Epitope Retrieval Solution becomes cloudy in appearance. 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 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 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.

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: Staining procedure**

After epitope retrieval, the slides placed in instrument racks are placed on the Dako Automated Link Platforms. The instrument will perform the staining process by applying the appropriate reagent, monitoring the incubation time and rinsing the slides between reagents. The reagent incubation times are preprogrammed in the Dako Link software.

**Step 3: Counterstain**

Slides may be counterstained with Hematoxylin for Automated Link Platforms (Code SK308). There are 2 staining protocols provided in the Dako software. One includes a hematoxylin counterstain and the other does not. The hematoxylin incubation time is preprogrammed in the protocol that includes a counterstain. See the User Guide for your Dako Automated Link Platform(s) for further information on programming protocols.

**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 Dako 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 (33) and reference 34 for additional information.

**Table 8: The purpose of daily quality control.**

<i>Tissue: Fixed and Processed Like Patient Sample</i>	<i>Specific Antibody and Detection System</i>	<i>Non-Specific Antibody* or Buffer Plus Same Detection System as Used with Specific Antibody</i>
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.

See the User Guide for your Dako Automated Link Platform(s) for further information on control slide programming.

### *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 a staining system in a diagnostic procedure, the user should verify the assay's performance by testing it on a series of in-house tissues with known IHC 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 (33). 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.

## **Staining interpretation - 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 "HercepTest™ Interpretation Manual – Gastric Cancer" for representative pictures of the staining intensities 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 Hoffmann et al. (49).

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  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 <b>ABSENCE</b> 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 labelling 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 (35). 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 labelling 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.

1. Evaluate the Control Cell Lines to validate the assay performance.
2. Evaluate the Positive and Negative Control Slides.
3. A hematoxylin and eosin (H&E) 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.
4. Evaluate the sections stained for HER2 protein overexpression at low power first. The majority of positive cases will be obvious at low power magnification.
5. For 1+ cases, use 40x objective magnification to verify membrane staining.
6. For 2+ cases, use 10x-20x objective magnification to verify membrane staining.

## **Surgical specimen**

1. Well-preserved and well-stained areas of the specimen should be used to make a determination of the percent of positive tumor cells.
2. If a majority of tumor cells demonstrate complete, basolateral or lateral membrane staining, the staining is either 2+ or 3+.
3. If there is complete, basolateral or lateral membrane staining at a strong intensity in equal to or more than 10% of the tumor cells in surgical specimens, the score of the specimen is 3+.
4. If there is complete, basolateral or lateral membrane staining at a weak to moderate intensity in equal to or more than 10% of the tumor cells in surgical specimens, the score of the specimen is 2+.
5. If equal to or more than 10% of the tumor cells in surgical specimens, stained only in part of their membrane, have a faint/barely perceptible intensity, the score of the specimen is 1+.
6. If no staining is observed the score of the surgical specimen is 0.
7. If less than 10% of the tumor cells in surgical specimens have staining, irrespective of the staining pattern (e.g. complete, basolateral, lateral or part of their membrane), the score is 0.

## **Biopsy specimen**

1. If there is a tumor cell cluster of at least 5 stained tumor cells with a strong complete, basolateral or lateral membrane staining, the score of the biopsy specimen is 3+, irrespective of percentage of tumor cells stained.
2. If there is a tumor cell cluster of at least 5 stained tumor cells with a weak to moderate complete, basolateral or lateral membrane staining, the score of the biopsy specimen is 2+, irrespective of percentage of tumor cells stained.
3. If there is a tumor cell cluster of at least 5 stained tumor cells with a faint/barely perceptible membrane staining and cells are stained only in part of their membrane, the score of the biopsy specimen is 1+, irrespective of percentage of tumor cells stained.
4. If no staining is observed the score of the biopsy specimen is 0.
5. If membrane staining (irrespective of staining intensity) is observed in less than 5 clustered tumor cells, the score of the biopsy specimen is 0.

## General limitations - Gastric

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 (36).
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 (37). Contact Dako Technical Support with documented unexpected reactions.
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) (37).
8. The staining procedure will be performed at the preprogrammed temperature (20–25 °C).

## Product specific limitations - Gastric

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) (38).
3. Do not replace kit reagents, with reagents carrying other lot numbers or with reagents from other manufacturers.
4. False results could be obtained from evaluation of cytoplasmic staining. Consider only the intensity of cell membrane staining when interpreting results.
5. 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.
6. 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.
7. Use of HercepTest™ on specimens fixed in fixatives other than neutral-buffered formalin has not been validated.
8. Note that normal tonsil and esophageal epithelia may stain up to 2+ intensity.
9. 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) (47, 48). 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 4.



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.**

	<b>FC N=296<sup>a</sup></b>	<b>FC+H N=298<sup>b</sup></b>
<b>FISH+ / IHC 0, 1+ subgroup (N=133)</b>		
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)
<b>Hazard ratio (95% CI)</b>	1.33 (0.92, 1.92)	
<b>FISH+ / IHC2+ subgroup (N=160)</b>		
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)
<b>Hazard ratio (95% CI)</b>	0.78 (0.55, 1.10)	
<b>FISH+ or FISH-/IHC3+<sup>c</sup> subgroup (N=294)</b>		
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)
<b>Hazard ratio (95% CI)</b>	0.66 (0.5, 0.87)	

Median survival was estimated from Kaplan-Meier curves.

- <sup>a</sup> Two patients on FC arm who were FISH+ but IHC status unknown were excluded from the analyses.
- <sup>b</sup> Five patients on Herceptin® arm who were FISH+ but IHC status unknown were excluded from the analyses.
- <sup>c</sup> 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**

*Intra-run reproducibility*

Intra-run reproducibility was tested in one laboratory with 3 specimens of different IHC staining scores. Each specimen was run in triplicate. This protocol was used with automated staining. All specimens gave 100% reproducible results. Intra-run reproducibility was tested in one laboratory with 11 specimens of different IHC staining scores. Each specimen was run in triplicate. This protocol was used with manual staining. All specimens gave 100% reproducible results.

*Inter-run and inter-laboratory 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 Agreements in Percent - A Subset of 12 Out of 60 Comparisons.**

		Observer 1		Observer 2		Average Agreement
		Agreement	CI95 LL <sup>1</sup>	Agreement	CI95 LL <sup>1</sup>	
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	

<sup>1</sup>CI95 LL: 95% lower limit confidence interval.

**Table 14. Site-to-site Overall Agreements in Percent.**

		Agreement	CI95 LL <sup>1</sup>	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	

<sup>1</sup>CI95 LL: 95% lower limit confidence interval.

Table 15. Observer-to-Observer Agreements in Percent.

		Agreement	CI95 LL <sup>1</sup>	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	

<sup>1</sup>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, ductal epithelium (2 of 3 tissues, 1+ staining intensity, >10% of cells, incomplete membrane)
Cervix uteri (3)	None
Colon (3)	None
Esophagus (3)	None
Heart (3)	None
Kidney (3)	None
Liver (3)	None
Lung (3)	None
Mesothelial cells (3)	None
Ovary (3)	None
Pancreas (3)	None
Parathyroid (3)	Chief cells (Oxyphil cells, 2 of 3 tissues, 1+ staining intensity, >50% of cells, membrane and cytoplasm)
Peripheral nerve (3)	None
Pituitary (3)	None
Prostate (3)	None
Salivary gland (3)	None
Skeletal muscle (3)	None
Skin (3)	Basal layer epithelium (2 of 3 tissues, 1-2+ staining intensity, >10% of cells, membrane and cytoplasm)
Small intestine (3)	None
Spleen (3)	None
Stomach (3)	Chief cells (1 of 3 tissues, 1-2+ staining intensity, >10% of cells, cytoplasm)
Testis (3)	None
Thymus (3)	None
Thyroid (3)	None
Tonsil (3)	None
Uterus (3)	Glandular epithelial cells (2 of 3 tissues, 0-1+ staining intensity, >50% of cells, cytoplasm)

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 (29) for remedial action, or contact Dako Technical Support Department to report unusual staining.

<i>Problem</i>	<i>Probable Cause</i>	<i>Suggested Action</i>
1. No staining of slides.	1a. Programming error. Reagents not used in proper order. 1b. Sodium azide in Wash Solution.  1c. Excessive heating of mounted tissue sections prior to deparaffinization and heat-induced antigen retrieval may lead to loss of visible HER2 immunoreactivity.	1a. Check slide log to verify that correct program was selected. 1b. Use only Dako Wash Buffer, Code S3006.  1c. 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 (27).
2. Weak staining of slides.	2a. Inadequate epitope retrieval.  2b. Inadequate reagent incubation times.  2c. Inappropriate fixation method 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.  2e. Insufficient reagent volume applied.	2a. Review the slide log to confirm that epitope retrieval was performed correctly. 2b. Review the slide log to confirm that reagent incubation times are correct. 2c. Ensure that patient tissue is not over-fixed or that an alternative fixative was not used. 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 (27). 2e. Check size of tissue section (22 mm x 22 mm) and reagent volume applied.
3. Excessive background staining of slides.	3a. Paraffin incompletely removed. 3b. Starch additives used in mounting sections to slides.  3c. Slides not thoroughly rinsed. 3d. Sections dried during staining procedure. 3e. Inappropriate fixation method used.  3f. Non-specific binding of reagents to tissue.	3a. Use fresh deparaffinization solutions. 3b. Avoid using starch additives for adhering sections to glass slides. Many additives are immunoreactive. 3c. Review the slide log to confirm that slides were rinsed properly. 3d. Verify that the appropriate volume of reagent is applied to slides. 3e. Ensure that approved fixative was used. Alternative fixative may cause excessive back-ground staining. 3f. 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. 5b. Reagent incubation times too long. 5c. Inappropriate wash solution used.	5a. Ensure that only approved fixatives and fixation methods are used. 5b. Review the slide log to confirm that reagent incubation times are correct. 5c. Use only Dako Wash Buffer, Code S3006.

<i>Problem</i>	<i>Probable Cause</i>	<i>Suggested Action</i>
6. Weak staining of the 1+ Control Slide Cell Line.	6a. Incorrect epitope retrieval protocol followed.	6a. Review the slide log to confirm that epitope retrieval was performed correctly.
	6b. Lack of reaction with Substrate-Chromogen Solution (DAB).	6b. Review the run log to confirm that the chromogen was prepared correctly. Review the slide log to confirm that the chromogen incubation time is correct.
	6c. Degradation of Control Slide.	6c. Check kit expiration date and kit storage conditions on outside of package.
7. Epitope Retrieval Solution is cloudy in appearance when heated.	7. When heated the solution turns cloudy in appearance.	7. This is normal and does not influence the staining.
8. Epitope Retrieval Solution is cloudy in appearance when stored (prior to heating).	8. The solution has been incorrectly stored or the expiration date of the solution has passed.	8. Check kit expiration date and kit storage conditions on outside of package. Discard the Epitope Retrieval Solution.














**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 Technical Support for further assistance. Additional information on staining techniques and specimen preparation can be found in Dako previously referenced Handbook (29) (available from Dako), Atlas of Immunohistology (39) and Immunoperoxidase Techniques. A Practical Approach to Tumor Diagnosis (40).

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**Explanation of symbols**

 Catalogue number	 Temperature limitation	 Batch code	 Fragile: handle with care
 In vitro diagnostic medical device	 Store in the dark	 Use by	 GHS pictogram ( <i>consult precautions section</i> )
 Consult instructions for use	 Contains sufficient for <n> tests	 Manufacturer	 GHS pictogram ( <i>consult precautions section</i> )
 Authorized representative in the European Community			







Agilent Technologies Singapore (International) Pte Ltd.  
No. 1 Yishun Avenue 7  
Singapore, 768923  
Tel. +44 161 492 7050  
[www.agilent.com](http://www.agilent.com)

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