P980018

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
2098 Gaither Road
Rockville MD 20850

Gretchen M. Murray, Ph.D., RAC
Assistant Manager, Regulatory Affairs
DAKO Corporation
6392 Via Real
Carpinteria, California 93013

SEP 25 1998

Re: P980018
DAKO HercepTest
Filed: May 18, 1998
Amended: June 2, June 4, August 4, August 10, August 18, August 24, August 31 and September 25, 1998.

Dear Dr. Murray:

The Center for Devices and Radiological Health (CDRH) of the Food and Drug Administration (FDA) has completed its review of your premarket approval application (PMA) for the DAKO Herceptest. This device is a semi-quantitative immunohistochemical assay to determine HER2 overexpression in breast cancer tissues routinely processed for histological evaluation. HercepTest is indicated as an aid in the assessment of patients for whom HERCEPTIN® (Trastuzumab) treatment is being considered (see Herceptin package insert). We are pleased to inform you that the PMA is approved subject to the conditions described below and in the "Conditions of Approval" (enclosed). You may begin commercial distribution of the device upon receipt of this letter.

The sale, distribution, and use of this device are restricted to prescription use in accordance with 21 CFR 801.109 within the meaning of section 520(e) of the Federal Food, Drug, and Cosmetic Act (the act) under the authority of section 515(d)(1)(B)(ii) of the act. FDA has also determined that, to ensure the safe and effective use of the device, the device is further restricted within the meaning of section 520(e) under the authority of section 515(d)(1)(B)(ii) insofar as the sale, distribution, and use must not violate sections 502(q) and (r) of the act.

In addition to the postapproval requirements in the enclosure, the postapproval reports must include the following information:

1. A protocol for a post approval study will be developed along with scientists from CDRH and CBER to determine clinical outcomes of patients selected for treatment with Herceptin by the DAKO HercepTest. The results of this post approval study will be reflected in the device labeling when the study is completed.

2. Your proposal for providing education and training material for users of the DAKO HercepTest should include photographs of examples of all four possible IHC scores, especially the 2+ IHC scores.

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Expiration dating for this device has been established and approved at 6 months when stored at 2-8°C. This is to advise you that the protocol you used to establish this expiration dating is considered an approved protocol for the purpose of extending the expiration dating as provided by 21 CFR 814.39(a)(8).

CDRH will notify the public of its decision to approve your PMA by making available a summary of the safety and effectiveness data upon which the approval is based. The information can be found on the FDA CDRH Internet HomePage located at http://www.fda.gov/cdrh/pmapage.html. Written requests for this information can also be made to the Dockets Management Branch, (HFA-305), Food and Drug Administration, 5630 Fishers Lane, Rm. 1061, Rockville, MD 20852. The written request should include the PMA number or docket number. Within 30 days from the date that this information is placed on the Internet, any interested person may seek review of this decision by requesting an opportunity for administrative review, either through a hearing or review by an independent advisory committee, under section 515(g) of the Federal Food, Drug, and Cosmetic Act (the act).

Failure to comply with the conditions of approval invalidates this approval order. Commercial distribution of a device that is not in compliance with these conditions is a violation of the act.

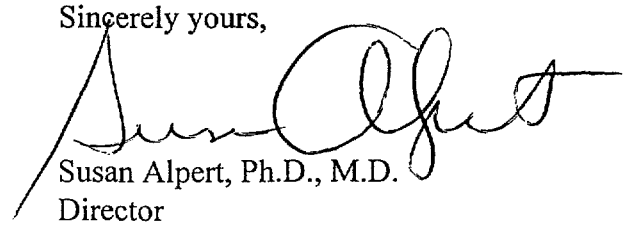
You are reminded that, as soon as possible and before commercial distribution of your device, you must submit an amendment to this PMA submission with copies of all approved labeling in final printed form.

All required documents should be submitted in triplicate, unless otherwise specified, to the address below and should reference the above PMA number to facilitate processing.

PMA Document Mail Center (HFZ-401)
Center for Devices and Radiological Health
Food and Drug Administration
9200 Corporate Blvd.
Rockville, Maryland 20850

If you have any questions concerning this approval order, please contact Peter E. Maxim, Ph.D. at (301) 594-1293.

Sincerely yours,



Susan Alpert, Ph.D., M.D.
Director
Office of Device Evaluation
Center for Devices and Radiological Health

Enclosure

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Issued: 3-4-98

CONDITIONS OF APPROVAL

APPROVED LABELING. As soon as possible, and before commercial distribution of your device, submit three copies of an amendment to this PMA submission with copies of all approved labeling in final printed form to the PMA Document Mail Center (HFZ-401), Center for Devices and Radiological Health, Food and Drug Administration (FDA), 9200 Corporate Blvd., Rockville, Maryland 20850.

ADVERTISEMENT. No advertisement or other descriptive printed material issued by the applicant or private label distributor with respect to this device shall recommend or imply that the device may be used for any use that is not included in the FDA approved labeling for the device. If the FDA approval order has restricted the sale, distribution and use of the device to prescription use in accordance with 21 CFR 801.109 and specified that this restriction is being imposed in accordance with the provisions of section 520(e) of the act under the authority of section 515(d)(1)(B)(ii) of the act, all advertisements and other descriptive printed material issued by the applicant or distributor with respect to the device shall include a brief statement of the intended uses of the device and relevant warnings, precautions, side effects and contraindications.

PREMARKET APPROVAL APPLICATION (PMA) SUPPLEMENT. Before making any change affecting the safety or effectiveness of the device, submit a PMA supplement for review and approval by FDA unless the change is of a type for which a "Special PMA Supplement-Changes Being Effected" is permitted under 21 CFR 814.39(d) or an alternate submission is permitted in accordance with 21 CFR 814.39(e). A PMA supplement or alternate submission shall comply with applicable requirements under 21 CFR 814.39 of the final rule for Premarket Approval of Medical Devices.

All situations which require a PMA supplement cannot be briefly summarized, please consult the PMA regulation for further guidance. The guidance provided below is only for several key instances.

A PMA supplement must be submitted when unanticipated adverse effects, increases in the incidence of anticipated adverse effects, or device failures necessitate a labeling, manufacturing, or device modification.

A PMA supplement must be submitted if the device is to be modified and the modified device should be subjected to animal or laboratory or clinical testing designed to determine if the modified device remains safe and effective.

A "Special PMA Supplement - Changes Being Effected" is limited to the labeling, quality control and manufacturing process changes specified under 21 CFR 814.39(d)(2). It allows for the addition of, but not the replacement of previously approved, quality control specifications and test methods. These changes may be implemented before FDA approval upon acknowledgment by FDA that the submission is being processed as a "Special PMA Supplement - Changes Being Effected." This acknowledgment is in addition to that issued by the PMA Document Mail Center for all PMA supplements submitted. This procedure is not applicable to changes in device design, composition, specifications, circuitry, software or energy source.

Alternate submissions permitted under 21 CFR 814.39(e) apply to changes that otherwise require approval of a PMA supplement before implementation of the change and include the use of a 30-day PMA supplement or annual postapproval report. FDA must have previously indicated in an advisory opinion to the affected industry or in correspondence with the applicant that the alternate submission is permitted for the change. Before such can occur, FDA and the PMA applicant(s) involved must agree upon any needed testing protocol, test results, reporting format, information to be reported, and the alternate submission to be used.

POSTAPPROVAL REPORTS. Continued approval of this PMA is contingent upon the submission of postapproval reports required under 21 CFR 814.84 at intervals of 1 year from the date of approval of the original PMA. Postapproval reports for supplements approved under the original PMA, if applicable, are to be included in the next and subsequent annual reports for the original PMA unless specified otherwise in the approval order for the PMA supplement. Two copies identified as "Annual Report" and bearing the applicable PMA reference number are to be submitted to the PMA Document Mail Center (HFZ-401), Center for Devices and Radiological Health, Food and Drug Administration, 9200 Corporate Blvd., Rockville, Maryland 20850. The postapproval report shall indicate the beginning and ending date of the period covered by the report and shall include the following information required by 21 CFR 814.84:

(1) Identification of changes described in 21 CFR 814.39(a) and changes required to be reported to FDA under 21 CFR 814.39(b).

(2) Bibliography and summary of the following information not previously submitted as part of the PMA and that is known to or reasonably should be known to the applicant:

> (a) unpublished reports of data from any clinical investigations or nonclinical laboratory studies involving the device or related devices ("related" devices include devices which are the same or substantially similar to the applicant's device); and
>
> (b) reports in the scientific literature concerning the device.

If, after reviewing the bibliography and summary, FDA concludes that agency review of one or more of the above reports is required, the applicant shall submit two copies of each identified report when so notified by FDA.

ADVERSE REACTION AND DEVICE DEFECT REPORTING. As provided by 21 CFR 814.82(a)(9), FDA has determined that in order to provide continued reasonable assurance of the safety and effectiveness of the device, the applicant shall submit 3 copies of a written report identified, as applicable, as an "Adverse Reaction Report" or "Device Defect Report" to the PMA Document Mail Center (HFZ-401), Center for Devices and Radiological Health, Food and Drug Administration, 9200 Corporate Blvd., Rockville, Maryland 20850 within 10 days after the applicant receives or has knowledge of information concerning:

(1) A mix-up of the device or its labeling with another article.

(2) Any adverse reaction, side effect, injury, toxicity, or sensitivity reaction that is attributable to the device and

(a) has not been addressed by the device's labeling or

(b) has been addressed by the device's labeling, but is occurring with unexpected severity or frequency.

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(3)Any significant chemical, physical or other change or deterioration in the device or any failure of the device to meet the specifications established in the approved PMA that could not cause or contribute to death or serious injury but are not correctable by adjustments or other maintenance procedures described in the approved labeling. The report shall include a discussion of the applicant's assessment of the change, deterioration or failure and any proposed or implemented corrective action by the applicant. When such events are correctable by adjustments or other maintenance procedures described in the approved labeling, all such events known to the applicant shall be included in the Annual Report described under "Postapproval Reports" above unless specified otherwise in the conditions of approval to this PMA. This postapproval report shall appropriately categorize these events and include the number of reported and otherwise known instances of each category during the reporting period. Additional information regarding the events discussed above shall be submitted by the applicant when determined by FDA to be necessary to provide continued reasonable assurance of the safety and effectiveness of the device for its intended use.

REPORTING UNDER THE MEDICAL DEVICE REPORTING (MDR) REGULATION. The Medical Device Reporting (MDR) Regulation became effective on December 13, 1984. This regulation was replaced by the reporting requirements of the Safe Medical Devices Act of 1990 which became effective July 31, 1996 and requires that all manufacturers and importers of medical devices, including in vitro diagnostic devices, report to the FDA whenever they receive or otherwise become aware of information, from any source, that reasonably suggests that a device marketed by the manufacturer or importer:

(1)May have caused or contributed to a death or serious injury; or

(2)Has malfunctioned and such device or similar device marketed by the manufacturer or importer would be likely to cause or contribute to a death or serious injury if the malfunction were to recur.

The same events subject to reporting under the MDR Regulation may also be subject to the above "Adverse Reaction and Device Defect Reporting" requirements in the "Conditions of Approval" for this PMA. FDA has determined that such duplicative reporting is unnecessary. Whenever an event involving a device is subject to reporting under both the MDR Regulation and the "Conditions of Approval" for a PMA, the manufacturer shall submit the appropriate reports required by the MDR Regulation within the time frames as identified in 21 CFR 803.10(c) using FDA Form 3500A, i.e., 30 days after becoming aware of a reportable death, serious injury, or malfunction as described in 21 CFR 803.50 and 21 CFR 803.52 and 5 days after becoming aware that a reportable MDR event requires remedial action to prevent an unreasonable risk of substantial harm to the public health. The manufacturer is responsible for submitting a baseline report on FDA Form 3417 for a device when the device model is first reported under 21 CFR 803.50. This baseline report is to include the PMA reference number. Any written report and its envelope is to be specifically identified, e.g., "Manufacturer Report," "5-Day Report," "Baseline Report," etc. Any written report is to be submitted to:

Food and Drug Administration
Center for Devices and Radiological Health
Medical Device Reporting
PO Box 3002
Rockville, Maryland 20847-3002

Copies of the MDR Regulation (FOD # 336&1336)and FDA publications entitled "An Overview of the Medical Device Reporting Regulation" (FOD # 509) and "Medical Device Reporting for Manufacturers" (FOD #987) are available on the CDRH WWW

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Home Page. They are also available through CDRH's Fact-On-Demand (F-O-D) at 800-899-0381. Written requests for information can be made by sending a facsimile to CDRH's Division of Small Manufacturers Assistance (DSMA) at 301-443-8818.

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P980018

SUMMARY OF SAFETY AND EFFECTIVENESS DATA

I. GENERAL INFORMATION

Device Generic Name:	DAKO Rabbit Anti-Human HER2 Immunohistochemistry Kit, for Immunoenzymatic Staining (Product Code No. K5204)
Device Trade Name:	DAKO HercepTest
Applicant's Name and Address:	DAKO A/S Produktionsvej 42 Glostrup, Denmark 011-45-44-920044
U.S. Contact:	DAKO Corporation 6392 Via Real Carpinteria, CA 93013
PMA Number:	P980018
Date of Panel Recommendation:	September 4, 1998
Date of Notice of Approval to the Applicant:	September 25, 1998

II. INDICATIONS FOR USE

DAKO HercepTest is a semi-quantitative immunohistochemical assay to determine HER2 overexpression in breast cancer tissues routinely processed for histological evaluation. HercepTest is indicated as an aid in the assessment of patients for whom HERCEPTIN® (Trastuzumab) treatment is being considered (see Herceptin package insert).

BACKGROUND

The human epidermal growth factor receptor 2, HER2 (also known as erbB2 or neu), gene encodes a protein product, referred to as p185 HER2 or HER2. The HER2 protein is a cell membrane receptor tyrosine kinase with homology to the epidermal growth factor receptor (EGRF or HER1).[1-8] The HER2 protein is normal component expressed by a variety of epithelial cell types, when the HER2 gene is not amplified. [8]

One consequence of HER2 gene amplification, however is an increase in HER2 protein expression on the surface of tumor cells.[9] Overexpression of the HER2 protein on the surface of breast cancer cells suggested that it could be a target for an antibody therapeutic. Trastuzumab

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(HERCEPTIN) 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 *in vitro & in vivo.*[11-13]

III. DEVICE DESCRIPTION

DAKO HercepTest was developed to provide an alternative to the investigational clinical trial assay (CTA) used in the Herceptin clinical studies. The performance of HercepTest was evaluated in an independent study comparing the results of the HercepTest to the CTA with 548 breast tumor specimens, none of which were from patients in the Herceptin clinical studies. HER2 overexpression was evaluated using HercepTest and the CTA. The results indicated a 79% concordance between the two assays on these tissue specimens. The concordance data also indicate that a 3+ reading with HercepTest is more likely to correspond with a positive reading on the CTA which would have met the entry criteria for the trial (2+ or 3+). Similarly, a 2+ reading on the HercepTest has a probability of corresponding to a 2+ result or a 0, 1+ result on the CTA, identifying populations that would receive limited or no benefit from Herceptin treatment.

HercepTest is an immunohistochemical test intended to aid in the assessment of patients being considered for Herceptin treatment. The test is interpreted and reported as negative for HER2 overexpression (0 and 1+ staining intensity) weakly reactive (2+ staining intensity) and strongly reactive (3+ staining intensity). In this manner DAKO HercepTest was shown to provide concordant results to the CTA used during the Herceptin trials.

CONTRAINDICATIONS, WARNINGS, PRECAUTIONS

Contraindications

- Use of the DAKO HercepTest is contraindicated for testing of non-cancerous tissues.
- Do not substitute primary antibodies or negative control reagents with primary antibodies and negative control reagents of different manufactured lots (lot numbers appear on vial labels).

Warnings

WARNING: The Herceptin clinical trials indicated that the degree of overexpression of HER2 was an important indicator of treatment effect. All of the patients in the Herceptin clinical trials were selected using a clinical trial assay. None of the patients in those trials were selected using the DAKO HercepTest. The DAKO HercepTest was compared to the clinical trial assay on an independent sample and found to provide acceptably concordant results. The actual correlation of the DAKO HercepTest to clinical outcome has not been established.

Other Warnings and Precautions for use of the device are stated in the product labeling.

IV. ALTERNATIVE PRACTICES AND PROCEDURES

There are several laboratory methods that can be used to measure HER2 overexpression. Fluorescence in situ hybridization (FISH) evaluates gene amplification directly in tissue sections. There is one test method utilizing FISH technology for which there is an approved PMA.

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V. MARKETING HISTORY

The DAKO HercepTest has not been marketed in the United States or any other country.

VI. POTENTIAL ADVERSE EFFECTS OF THE DEVICE ON HEALTH

Patients falsely assigned as positive (3+, 2+) following initial assessment of HER2 status with the DAKO HercepTest would be considered for treatment with Herceptin. The benefit of Herceptin therapy to patients with normal or low level of HER2 overexpression is unknown. The risks of Herceptin treatment, while not negligible, compare with that of standard cytotoxic chemotherapy.

Patients falsely assigned as negative (0, 1+) following initial assessment of HER2 status with the DAKO HercepTest would not be considered eligible for treatment with Herceptin.

VII. SUMMARY OF STUDIES

A. Preclinical Studies

Preclinical testing of the DAKO HercepTest included selection, verification, and optimization of device reagents, reagent stability, HER2 antigen stability, characterization of control cell lines, specificity of HER2 antibody, and reproducibility studies.

1. Selection, Verification, and Optimization of the Kit Contents.

   The objective of these studies was to optimize test performance. Each step of the immunohistochemical procedure was tested using two or more possible alternatives to determine the optimal formulation of the individual components. The following results were obtained:

   - The antigen retrieval step was investigated using several different protocols. Pretreatment by proteolytic digestion, microwave and heat-induced epitope retrieval were compared. It was determined that heat-induced epitope retrieval using the water bath method provided the most reproducible results.

   - Two different commercially available visualization systems, LSAB+ and EnVision+, were tested. EnVision required fewer steps in the process, and the EnVision polymer did not have any biotinylation step, which reduced the potential for background staining due to endogenous biotin. The EnVision system was selected due to design criteria.

   - The wash buffer component was tested to select the optimal buffer.

2. Device Stability.

   The objective of this study was to determine the expiration date of the kit. The HercepTest was stored under the following conditions: 20° and 37°C; three each freeze-thaw cycles, followed by testing; heating to 37°C for 24 hours followed by storage at 10°C; and 3 freeze-thaw cycles followed by storage at 10°C. Three lots of the device were tested by this procedure. The kits were tested at intervals of 2, 4, and 8 weeks. Results of this testing indicated that the test kit was stable for up to 8 weeks at 37°C. This was indicative of a projected shelf life for the kit of 6 months at 2° - 8°C.

3. HER2 antigen stability.

   The objective of this study was to determine the stability of the HER2 antigen in freshly cut tissue sections. Two paraffin embedded control cell line pellets and two well-characterized breast cancer tumors were tested at days 1, 15 and 43. The tissue sections had been cut and stored in the dark at room temperature (20°C). The HER2 expression was evaluated by immunohistochemical (IHC) procedures. No change in epitope responsiveness was noted during the 43-day holding period for these specimens. This study showed that freshly cut sections could be used up to 6 weeks when stored at room temperature in the dark.

4. Characterization of control cell lines.

   The three cell line controls were characterized with regard to the number of protein receptors on the cell surface. Scatchard analysis of the number of cell receptors was performed at Genentech, Inc., South San Francisco, CA. Results are presented in the Table 1.

Table 1
Number of HER2 Receptors on
Breast Cancer Cell Lines Used as Standards

Cell Line	IHC Score	Receptors/ Cell
MDA-231	0	21,600 ± 6,700
MDA-175	1+	92,400 ± 12,000
SK-BR3	3+	2,390,000 ± 130,000

[a] **Receptor numbers were derived by Scatchard analysis using iodinated 4D5 antibody carried**

These studies provided the link between the DAKO Herceptin Test and molecular characterization of the specimens.

5. Antibody Specificity Studies

The specificity of the HercepTest primary antibody for the HER2 protein with regard to cross reactivity with HER1 (epidermal growth factor receptor) and HER3 and 4 was demonstrated with Western blot analysis using stable transfectants of established cell lines.

The specificity of the HercepTest for use with metastatic breast tumor tissue obtained from elsewhere in the body was tested in a variety of normal tissues to evaluate unexpected background staining. Results are presented in Table 2.

Table 2
Summary of HercepTest Normal Tissue Reactivity

TISSUE TYPE (# tested)	POSITIVE TISSUE ELEMENT
Adrenal (3)	None
Bone Marrow (3)	None
Brain/Cerebellum (3)	None
Brain/Cerebrum (3)	None
Breast (3)	Mammary gland (1+ staining intensity)
Cervix (3)	None
Colon (3)	Columnar epithelium, surface (1+ staining intensity)
Esophagus (3)	Squamous epithelia (1/3 tissues, 2+ staining intensity)
Heart (3)	None
Kidney (3)	Tubule (1+ staining intensity)
Liver (3)	None
Lung (3)	None
Mesothelial Cells (3)	None
Ovary (3)	None
Pancreas (3)	Langerhans cells, Cytoplasmic (3+ staining intensity)
Parathyroid (3)	None
Pericardium (1)	None
Peripheral Nerve (3)	None
Pituitary (3)	Endocrine cells (Cytoplasmic, 3+ staining intensity)
Prostate (3)	Prostate gland (2+ staining intensity)
Salivary Gland (3)	None
Skeletal Muscle (3)	None
Skin (3)	None
Small Intestine (3)	Columnar epithelium, surface (1+ staining intensity)
Spleen (3)	None
Stomach (3)	Epithelium (1/3 tissues, 1+ staining intensity)
Testis (3)	None
Thymus (3)	None
Thyroid (3)	None
Tonsil (3)	Squamous epithelia (2+ staining intensity)
Uterus (3)	Endometrium (1/3 tissues, 1+ staining intensity)

6. Reproducibility Studies

The reproducibility studies were designed to characterize intra-run, inter-run, inter-laboratory and between lot reproducibility, and reproducibility between manual and automated staining methods.

INTRA-RUN REPRODUCIBILITY STUDY

Intra-run reproducibility was tested in one laboratory with 5 specimens of different IHC intensity staining scores. Each specimen was run in a masked randomized format in triplicate with a corresponding slide tested with the negative reagent control to assess background staining. This protocol was used with manual staining and again with automated staining with 5 different tissues. This study was performed at Impath Laboratories of Los Angeles, CA. All specimens

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using both manual and automated techniques gave reproducible positive vs. negative results. All 2+ and 3+ IHC scores were also 100% reproducible indicating intra-run reproducibility at Impath Laboratories.

INTER-RUN REPRODUCIBILITY STUDY

Inter-run reproducibility was tested at three laboratories. Each of five specimens was paired with its corresponding slide tested with the negative reagent control to assess background staining. These five pairs of slides were randomized and masked from run to run. Satisfactory reproducibility was seen for positive vs. negative results (0 and 1+ vs. 2+ and 3+). There was no irreproducibility seen in distinguishing 2+ from 3+ in any of the specimens in these studies.

INTER-LABORATORY REPRODUCIBILITY STUDY (INCLUDING MANUAL VS. AUTOMATED METHODOLOGIES)

There were two objectives of the Inter-Laboratory Reproducibility Study. The first was to investigate the reproducibility of the DAKO HercepTest between laboratories using freshly cut sections from the same specimens. The second was to investigate the reproducibility of the DAKO HercepTest between manual and automated techniques in each of the laboratories using freshly cut sections from the same specimens. Inter-laboratory reproducibility was studied at 6 geographically separated laboratories with 40 identical randomized and masked specimens of various IHC intensity scores. Each section was tested with the primary antibody and the negative reagent control to assess background staining. The freshly cut sections were forwarded to each testing laboratory for manual and automated staining and evaluation by a pathologist.

Three of the 6 laboratories that participated in the inter-laboratory reproducibility studies had valid control results. The agreement of these 3 laboratories with each other's results ranged from 82% to 90% for a dichotomous positive/negative determination where 0 and 1+ were negative and 2+ and 3+ were positive for overexpression of HER2. This range included both manual and automated staining methods. A total of 15 discrepant results of 120 sample comparisons were seen. An additional 12 of the 120 samples had discrepant results between 2+ and 3+ staining intensity. No difference could be discerned between manual and automated staining reproducibility.

The 3 laboratories that had invalid control results identified areas of the product labeling that needed strengthening to prevent failure of the controls when the comes to the market place. One laboratory used a microwave oven in place of the recommended water bath for the antigen retrieval step. It was decided that methods of antigen retrieval other than the use of a 95-99°C water bath should not be recommended. Another laboratory did not allow the temperature of the water bath to recover to the specified temperature of 95 - 99°C before staring to time the incubation during the antigen retrieval step. The package insert instructions were changed to address this factor. The reason for the failed control in the third laboratory was not specified.

LOT-TO-LOT REPRODUCIBILITY STUDY

The objective of the Lot-to-Lot Reproducibility Study, which was performed at DAKO A/S of Glostrop, Denmark, was to demonstrate that comparable and reproducible results could be obtained with 3 lots of the device. Each new lot of HercepTest was run on the same specimens as the previous lot. The testing was conducted on the following formalin-fixed paraffin embedded specimens: the 0, 1+, and 3+ cell line controls; two different breast carcinomas expressing the HER2 receptor; and a normal HER2-negative tissue (tonsil). All three lots had identical results for the control cell lines and the HER2 negative tissue. One lot was off by one

IHC intensity score on the two breast carcinoma samples. One carcinoma remained positive with this variance, but the other varied between a positive (2+) and a negative result.

B. Clinical Studies

LabCorp (Research Triangle Park, NC) was the central laboratory used to screen potential patients for the Herceptin™ clinical trials using an investigational immunohistochemical (IHC) clinical trial assay (CTA). The DAKO HercepTest was investigated as a suitable alternative to the CTA. Studies were undertaken using independent clinical specimens to establish the performance characteristics of the DAKO HercepTest and its concordance with the CTA: a concordance study comparing HercepTest results to CTA and comparison to results of well-characterized tissues.

STUDY OBJECTIVE:

The objective of this study was to compare HER2 overexpression as determined by the DAKO HercepTest compared to that of the CTA. A goal of at least 75% concordance with 95% confidence was selected before it would be acceptable to substitute the DAKO HercepTest for the CTA. Concordance or agreement was defined as the total number of specimens that agreed with the CTA results divided by the total number of samples tested.

STUDY PROTOCOL

Breast sample specimens were obtained from the National Cancer Institute Cooperative Breast Cancer Tissue Resource. These were screened by the CTA. All positive specimens were included in the concordance study. An equal number of negative samples were randomly selected from the remaining negative samples. It was projected that there would be about 30% of specimens positive for HER2 antigen; 300 positives were expected. These 300 positive and 300 negative specimens would be tested by the DAKO HercepTest and concordance of results to the CTA calculated. Concordance or agreement between the two assays was equal to the number of specimens positive by both tests and negative by both tests divided by the total number of samples chosen to be in the concordance study.

INVESTIGATOR

All samples were tested at LabCorp of Research Triangle Park, North Carolina. The principle investigator was Steven Anderson, Ph.D., Director of Oncology and Infectious Diseases.

STUDY POPULATION

The 1190 specimens obtained from the National Cancer Institute Cooperative Breast Cancer Tissue Resource were screened by LabCorp using the CTA to find 274 positive specimens, which constituted 23% of the 1190 specimens. The percentages of 0,1+, 2+, and 3+ specimens in this sampling also differed from those seen in the Herceptin® clinical trial. All 274 positive specimens were included in the concordance study. Then an equal number of negative samples were randomly selected from the remaining negative samples.

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RESULTS

Table 3
Results of Concordance Study

		Clinical Trial Assay +	Clinical Trial Assay -	
DAKO	+	216	**59**	275
	-	**58**	215	273
		274	274	548

Concordance = 79% (76 - 82%) 95% Confidence Interval

The overall binary concordance of the DAKO HercepTest System to the CTA was 79% (431/548), with a 2-sided 95% confidence interval of 76% -82%. An equivalent number of positive and negative results were obtained by both methods. However, 22% of results were discordant between these two methods. The overall level of agreement was 79%, which was statistically significantly higher than the predetermined unacceptable level ($p = 0.0153$). The two-sided 95% confidence interval was 76% to 82%. This level of concordance indicated that the DAKO HercepTest results and the CTA results were similar.

Table 4
Concordance Data for DAKO HercepTest Kit and Clinical Trial Assay

		Clinical Trial Assay		
		3+	2+	0 - 1+
HercepTest	3+	107	36	6
	2+	16	57	53
	0 - 1+	8	50	215

The concordance data in Table 4 indicates that a 3+ reading on the HercepTest was likely to correspond with a positive result on the clinical trial assay which would have met the entry criteria for the trial (2+ or 3+). A 2+ reading on the HercepTest has a probability of corresponding with a result on the clinical trial assay which either would not have allowed study entry (0 - 1+) or with a result (2+) identifying a population for which evidence suggesting treatment benefit is limited.

HercepTest Results with Well-characterized Breast Cancer Tissues (Accuracy)

STUDY OBJECTIVE

The objective of this study was to determine the agreement between the DAKO HercepTest and the HER2 "status" of well-characterized tissues.

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INVESTIGATOR

All testing, including the DAKO HercepTest, was performed in the laboratory of Dr. Michael Press, MD, Ph.D. ,at the University of Southern California (USC) School of Medicine, Dept. of Pathology, Los Angeles, CA.

STUDY POPULATION

The DAKO test was performed on 2 microscope slides containing randomly located paraffin-embedded tissue sections from 168 breast tumors. These tumors had been-previously characterized by five different methods for determination of overexpression of HER2 receptors, including in-house Southern blot and FISH for amplification of DNA, Northern blot RNA analysis, and Western blot and immunohistochemistry (IHC) on frozen tissues for protein overexpression.

Table 5
Comparison of DAKO IHC to
Molecular Tests for Gene Amplification & Overexpression (OE)

		Reference OE Classification		
		+	-	
DAKO	+	43	0	43
	-	26	99	125
		69	99	168

Positive Agreement = 43/69 = 62%
Negative Agreement = 99/99 = 100%

Concordance = 84.5% (78 - 89%)

The results indicated an 85% (142/168) level of agreement (95% confidence interval of 78-89%) between the degree of positivity (2+ and 3+) and negativity (0 and 1+ DAKO IHC score) determined using the DAKO kit and some degree of overexpression by Dr. Press's method of classification of overexpression (Table 5).

VIII. CONCLUSIONS DRAWN FROM THE STUDIES

The clinical studies conducted on the DAKO HercepTest demonstrated: 1) that results were concordant with the CTA used to screen potential patients for the HERCEPTIN clinical trials, 2) that HerecepTest had an acceptable comparison to assessments of various molecular abnormalities of HER2 overexpression, and 3) that HercepTest results were reproducible within and across laboratories.

RISK-BENEFIT ANALYSIS

Testing has been completed to characterize the performance of the DAKO HercepTest relative to the CTA. There was a sufficient level of concordance between the two assay to expect that the clinical experience with Herceptin is limited to patients with a 2+ or 3+ staining intensity score using the CTA, and could be obtained with tissues considered "positive" in the DAKO HercepTest.

The risk of false positive results could allow a breast cancer patient whose tumor did not overexpress HER2 to be treated with Herceptin. The benefits, if any, of Herceptin treatment in this patient population are unknown.

False negative test results would potentially exclude the patient from treatment with Herceptin. Because all clinical experience with Herceptin was derived from treatment of patients with 2+ or 3+ levels of overexpression by the CTA, exclusion of patients with lower levels of overexpression was considered appropriate.

SAFETY

The safety of the DAKO HercepTest is acceptable, as it is used on an in vitro basis. One hazardous material (the DAB chromogen) included in the system requires safety labeling. At the concentration of chromogen that is present in the liquid waste from the assay, this material has been diluted to a non-hazardous concentration. Instructions for safe handling of the concentrated chromogen are included in the package insert. These safety precautions should be sufficient to protect technicians using the DAKO Anti-HER2 IHC System.

EFFECTIVENESS

The testing that has been completed by DAKO for the concordance of the DAKO HercepTest to the CTA indicated that the DAKO HercepTest performed in a similar manner to the CTA, would identify a patient population eligible for Herceptin therapy, and was reproducible within and across laboratories. These results supported the intended use claim included in the package insert.

IX. PANEL RECOMMENDATIONS

A public meeting of the combined FDA Hematology and Pathology Devices and Immunology Devices Advisory Panel was conducted on September 4, 1998 to consider PMA. The panel gave a recommendation of approvable with the following conditions:

1. A protocol for a post approval study will be developed along with scientists from The Center for Devices and Radiological Health (CDRH) and the Center for Biologics Evaluation and Research (CBER) to determine clinical outcomes of patients selected for treatment with Herceptin by the DAKO HercepTest. The results of this post approval study will be reflected in the device labeling when the study is completed.

2. The proposal for providing education and training material for users of the DAKO HercepTest should include photographs of examples of all four possible IHC scores, especially the 2+ IHC scores.

3. Draft labeling containing the following revisions:

   a. Clarification of the numerical IHC scores (0,1,2,3+) with regard to results obtained in the Herceptin treatment trials.

   b. Definition of the Procedure as much as possible to improve test reproducibility. For example, specify specific incubation and antigen retrieval time periods.

   c. Emphasis on adherence to the specified test procedure to maintain and improve assay reproducibility, including the importance of performing antigen retrieval using only a

calibrated water bath. Provide special instructions for specific instances, such as, differences in boiling point and corresponding water bath temperature at high elevations.

d. Provide a list of allowable sample fixatives; whether any special procedures must be performed to retrieve antigen with allowable fixatives; and how to ensure accurate staining with all allowable fixatives.

e. Description of expected sample aging (cut and uncut paraffin embedded specimens).

IX. FDA DECISION

CDRH issued an approval order for the applicant's HercepTest on September 25, 1998.

The applicant's manufacturing and control facilities were inspected on September 24, 1998 and the facilities were found to be in compliance with the Good Manufacturing Practice Regulations (GMPs). The shelf-life of DAKO's Anti-HER2 IHC System has been established at 6 months when stored at 2-8° C.

XI. APPROVAL SPECIFICATIONS

Directions for use: See labeling

Conditions of Approval: CDRH approval of this PMA is subject to full compliance with the conditions described in the approval order.

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PART 8: LABELING

PACKAGE INSERT

DAKO HercepTest™ for Immunoenzymatic Staining Code No. K5204
Intended Use: For in vitro diagnostic use

DAKO HercepTest™ is a semi-quantitative immunohistochemical assay to determine HER2 overexpression in breast cancer tissues routinely processed for histological evaluation. HercepTest™ is indicated as an aid in the assessment of patients for whom HERCEPTIN® (Trastuzumab) treatment is being considered (See HERCEPTIN® package insert).

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

SUMMARY AND EXPLANATION

Background

The human *HER-2* gene (also known as *ERBB2* or *NEU*) encodes a protein often referred to as HER2 protein or $p185^{HER2}$. The HER2 protein is a membrane receptor tyrosine kinase with homology to the epidermal growth factor receptor (EGFR or HER1).[1-8] The HER2 protein is a normal component expressed by a variety of epithelial cell types.[8]

In a fraction of patients with breast cancer the HER2 protein is overexpressed as part of the process of malignant transformation and tumor progression.[9] Overexpression of the HER2 protein on the surface of breast cancer cells suggested that it could be a target for an antibody therapeutic. Trastuzumab (HERCEPTIN®) 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]

HercepTest Characteristics

DAKO 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 indicate 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

The DAKO HercepTest™ contains reagents required to complete a two step immuno-histochemical 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 antibody 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. 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™ is applicable for both manual and automated staining.

REAGENTS PROVIDED

The following materials, sufficient for 35 tissue sections based upon 100 µL of DAKO Ready-to-Use Rabbit Anti-Human HER2 Protein per tissue section, are included:

Vial No.	Quantity	Description
1	1 × 7 mL	Peroxidase-Blocking Reagent: 3% hydrogen peroxide containing 0.2% sodium azide (NaN_3).
2	1 × 3.5 mL	**Rabbit Anti-Human HER2 Protein: Ready-to-use affinity-isolated** antibody supplied in 0.05 mol/L Tris/HCl, 0.1 mol/L NaCl, 15 mmol/L NaN_3, pH 7.2, containing stabilizing protein. Immunogen: Synthetic C-terminal fragment (intracytoplasmic part) of the HER2 protein coupled to keyhole limpet haemocyanin. Specificity: HER2 ~~onco~~protein. Purification method: **The antibody** is affinity isolated by using an immobilized HER2 ~~onco~~protein peptide.
3	1 × 7 mL	Visualization Reagent: Dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-rabbit immunoglobulins in Tris-HCl buffer containing stabilizing protein and an anti-microbial agent.
4	1 × 3.5 mL	Negative Control Reagent: Immunoglobulin fraction of normal rabbit serum, at an equivalent protein concentration as the antibody to HER2 protein. Supplied in 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, 15 mmol/L NaN_3, pH 7.2, containing stabilizing protein.
5	1 × 7 mL	DAB Buffered Substrate: Substrate buffer solution, pH 7.5, containing hydrogen peroxide, stabilizers, enhancers and an anti-microbial agent.
6	1 × 0.5 mL	DAB Chromogen: 3,3′-diaminobenzidine chromogen solution.
7	1 × 250 mL	Epitope Retrival Solution (x 10): Citrate buffer with an anti-microbial agent.
8	1 × 250 mL	Wash Buffer (x 10): Tris-HCl buffer with a detergent and an anti-microbial agent.
	5 slides	Control Slides: Each slide contains sections of three formalin-fixed, paraffin-embedded breast carcinoma cell lines representing different levels of HER2 protein expression: MDA-231 (0+), MDA-175 (1+) and SKBR3 (3+).

Materials Required but Not Supplied

Absorbent wipes

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

Counterstain: hematoxylin, such as aqueous based, Mayer's hematoxylin, DAKO® Code No. S 3309 (see Instructions For Use, A.4)

Coverslips

Distilled or deionized water (Washing water)

Drying oven, capable of maintaining 60 °C or less.

Ethanol, 70% and 95%

Humid chamber (optional)

Light microscope (20x–800x)

Mounting media, such as DAKO® Faramount (Code No. S 3025) or DAKO Glycergel® (Code No. C 0563)

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

Slides, SuperFrost Plus or Poly-L-lysine coated or DAKO® Silanized Slides (Code No. S 3003) (see Specimen Preparation)

Staining jars or baths

Timer (capable of 2–40 minute intervals)

Wash bottles

Waterbath (Capable of maintaining 95-99 °C temperature range)

Xylene, toluene, or xylene substitutes

STORAGE

Store at 2–8 °C.

DAKO HercepTest is suitable for use 6 months from the date of manufacture when stored at 2-8 °C. Do not use after the expiration date stamped on the package. If reagents are stored under any conditions other than those specified in the package insert, they must be validated by the user.[14]

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 Technical Services.

SPECIMEN PREPARATION

Specimens from biopsy must be handled to preserve the tissue for IHC staining. Standard methods of tissue processing should be used for all specimens.[15]

Paraffin Embedded Sections: Tissues preserved in neutral buffered formalin or Bouin's for routine processing and paraffin embedding, are suitable for use. For example, specimens from the biopsy should be blocked into a thickness of 3 or 4 mm and fixed for 18–24 hours in neutral buffered formalin. The tissues are then dehydrated in a series of alcohols and xylene, followed by infiltration by melted paraffin held at no more than 60 °C. Properly fixed and embedded tissues expressing the HER2 protein will keep indefinitely prior to sectioning and slide mounting if stored in a cool place (15-25 °C).[15,16] The Clinical Laboratory Improvement Act of 1988 requires in 42 CFR 493.1259(b) that "The laboratory must retain stained slides at least ten years from the date of examination and retain specimen blocks at least two years from the date of examination."[16]

The slides required for HER2 protein evaluation and 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, 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).[17]

Consult the DAKO® *Handbook: Immunohistochemical Staining Methods*[18] or references 15 and 16 for further details on specimen preparation.

Treatment of Tissues Prior to Staining: A specific epitope retrieval method, boiling in 10 mmol/L citrate buffer, must be used for optimal assay performance. The epitope retrieval solution is supplied in the HercepTest™ kit. This procedure involves heating of tissue sections mounted on slides that are immersed in 10 mmol/L citrate buffer[17] in a calibrated waterbath capable of maintaining the required temperature (95–99 °C). Laboratories located at higher elevations should determine the best method of maintaining the required waterbath temperature. Other methods of heating have been tested and do not give reproducible results. Immediately after epitope retrieval, commence the staining procedure. Deviation from the described procedure may affect results.

PRECAUTIONS

1. For in vitro diagnostic use.
2. 3,3'-diaminobenzidine (DAB) may be harmful by inhalation, in contact with skin and if swallowed. Material is irritating to eyes and skin. If skin contact should occur, flush affected area with soap and water. NOTE: Although diaminobenzidine is structurally related to benzidine, there is no evidence for the carcinogenicity of diaminobenzidine. Consult Federal, State or local regulations for disposal.
3. This product contains sodium azide (NaN_3) , a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, build-ups of sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide build-up in plumbing.[20,21]
4. 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.[22] 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.
5. Minimize microbial contamination of reagents to avoid nonspecific staining.
6. Incubation times, temperatures, or methods other than those specified may give erroneous results.
7. Reagents have been optimally diluted. Further dilution may result in loss of antigen staining.

8. Do not substitute primary antibodies or negative control reagents with primary antibodies and negative control reagents of different manufactured lots (lot numbers appear on vial labels).
9. The Visualization Reagent and DAB+ Chromogen may be affected adversely if exposed to excessive light levels. Do not store system components or perform staining in strong light, such as direct sunlight.
10. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin. Refer to the Material Safety Data Sheet (MSDS) for additional information.

INSTRUCTIONS FOR USE

A. REAGENT PREPARATION

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

A 1 Epitope Retrieval Solution

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

A.2 Wash Buffer Solution

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

Distilled or deionized water may be used for rinsing the Peroxidase Blocking Reagent, Substrate-Chromogen, and counterstain.

A.3 Substrate-Chromogen Solution (DAB)

The following procedure yields 1 mL of Substrate-Chromogen solution. Each 1 mL aliquot is sufficient for ten tissue sections.

Step 1:	Transfer 1 mL of DAB+ Buffered Substrate from Vial 5 to a test tube.
Step 2:	Add one drop (25–30 µL) of DAB+ Chromogen from Vial 6. Mix and apply to tissue sections with a pipette.

Prepared Substrate-Chromogen solution 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.

A.4 Counterstain

The colored end-product of the staining reaction is alcohol and water insoluble. Use a hematoxylin counterstain and adjust the hematoxylin staining intensity to a similar level shown in the HercepTest™ Atlas. Hematoxylin, either alcohol or aqueous-based such as Mayer's hematoxylin (DAKO Code No. S 3309) may be used. Follow hematoxylin counterstaining with a thorough rinse in distilled water, then immerse tissue slides into a bath of 37 mmol/L ammonia water (see Section B. Step 6). Ammonia water (37 mmol/L) is prepared by mixing 2.5 mL of 15 mol/L (concentrated) ammonium hydroxide with 1 liter of distilled or deionized water. Unused 37 mmol/L ammonia water may be stored at room temperature (20–25 °C) in a tightly capped bottle for up to 12 months.

A.5 Mounting Media

Non-aqueous, permanent mounting medium is recommended. However, aqueous mounting is also acceptable. DAKO® Faramount Aqueous Mounting Medium, Ready-to-Use (Code No. S 3025) or DAKO Glycergel® Mounting Medium (Code No. C 0563) is recommended for aqueous mounting. Liquefy DAKO Glycergel® by warming to approximately 40 °C ± 5 °C prior to use.

B. STAINING PROCEDURE

B.1 Procedural Notes

The user should read these instructions carefully and become familiar with all components prior to use. (See PRECAUTIONS.)

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

Do not allow tissue sections to dry during the staining procedure. Dried tissue sections may display increased nonspecific staining. Cover slides exposed to drafts. If prolonged incubations are used, place tissues in a humid environment.

If the staining procedure must be interrupted, slides may be kept in a buffer bath following incubation of the primary antibody (Step 2) 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 nonspecific 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 95–96% ethanol for 3 (±1) minutes. Change baths and repeat once.
3. Tap off excess liquid and place slides in 70% ethanol for 3 (±1) minutes. Change baths and repeat once.
4. Tap off excess liquid and place slides in distilled or deionized water for a minimum of 30 seconds. Commence staining procedure as outlined in Section B.2 with Epitope Retrieval.

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

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

B.2 Staining Protocol (Performed at Room Temperature, 20–25 °C)

STEP 1: EPITOPE RETRIEVAL

Place Coplin jars in a waterbath, then fill jars with the diluted Epitope Retrieval Solution (see Instructions for Use, Section A.1.) and heat waterbath and the Epitope Retrieval Solution to 95–99 °C.

Immerse the room temperature deparaffinized sections into the preheated buffer in the Coplin jars. Bring temperature back to 95–99 °C and then incubate for 40 ± 1 minutes at 95–99 °C.

Remove the entire jar with slides from the waterbath. Allow the slides to cool in the buffer for 20 ± 1 minutes at room temperature.

Decant the buffer and rinse in the Wash Buffer. (See Instructions for Use, Section A .2.)

STEP 2: PEROXIDASE-BLOCKING REAGENT

Tap off excess buffer. Using a lintless tissue (such as a Kimwipe or gauze pad), carefully wipe around the specimen to remove any remaining liquid and to keep reagents within the prescribed area.

Apply enough Peroxidase-Blocking Reagent to cover specimen.

Incubate 5 (±1) minutes.

Rinse *gently* with distilled or deionized water or Wash Buffer from a wash bottle (do not focus the flow directly on tissue) and place in a fresh buffer bath.

STEP 3: PRIMARY ANTIBODY OR NEGATIVE CONTROL REAGENT

Tap off excess buffer and wipe slides as before.

Cover specimen with 100 uL of primary antibody or Negetaive Control Reagent.

Incubate 30 (±1) minutes.

Rinse *gently* with Wash Buffer from a wash bottle (do not focus the flow directly on tissue) and place in a fresh buffer bath.

If the staining procedure must be interrupted, slides may be kept in Wash Buffer following incubation of the primary antibody (Step 3) for up to one hour at room temperature (20–25 °C) without affecting the staining performance.

STEP 4: VISUALIZATION REAGENT

Tap off excess buffer and wipe slides as before.

Cover specimen with 100 uL of Visualization Reagent.

Incubate 30 (±1) minutes.

Rinse slides as in Step 3.

STEP 5: SUBSTRATE-CHROMOGEN SOLUTION (DAB)	
	Wipe slides as before.
	Cover specimen with 100 uL of the Substrate-Chromogen Solution.
	Incubate for 10 (± 1) minutes.
	Rinse *gently* with distilled or deionized water from a wash bottle (do not focus flow directly on tissue). Collect Substrate-Chromogen Solution waste in a hazardous materials container for proper disposal.
STEP 6: COUNTERSTAIN (Directions are for HEMATOXYLIN)	
	Immerse slides in a bath of hematoxylin. Incubate for 2–5 minutes, depending on the strength of hematoxylin used.
	Rinse *gently* in a distilled or deionized water bath.
	Optional: Dip slides 10 times into a bath of 37 mmol/L ammonia water. (See Section A.4.)
	Rinse slides in a bath of distilled or deionized water for 2–5 minutes.
	NOTE: Depending on the incubation length and potency of the hematoxylin used, counterstaining will result in a pale to dark blue coloration of the cell nuclei. Excessive or incomplete counterstaining may compromise proper interpretation of results.
STEP 7: MOUNTING	
	Non-aqueous, permanent mounting media are recommended. Otherwise, aqueous mounting media are also acceptable. Specimens may be mounted and coverslipped with an water-based mounting medium such as DAKO® Faramount (Code No. S 3025) or DAKO Glycergel® (Code No. C 0563).
	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

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 DAKO-supplied control slides. Consult the quality control guidelines of the College of American Pathologists (CAP) Certification Program for Immunohistochemistry, NCCLS Quality Assurance for Immunocytochemistry, Proposed Guideline,[23] and reference 24 for additional information.

Table 1
The Purpose of Daily Quality Control

Tissue: Fixed and Processed Like Patient Sample	Specific Antibody and Secondary Antibody	Nonspecific Antibody[a] or Buffer Plus Same Secondary Antibody As Used With Specific Antibody
Positive Control: Tissue or cells containing target antigen to be detected (could be located in patient tissue). The ideal control is weakly positive staining tissue to be most sensitive to antibody or antigen degradation.	Controls all steps of the analysis. Validates reagent and procedures used for HER2 staining	Detection of nonspecific 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 nonspecific background staining
Patient Tissue	Detection of specific staining	Detection of nonspecific background staining
DAKO-supplied Control Slide	Controls staining procedure only	

[a] Same source and type as the specific antibody, but not directed against the same target antigen. To detect nonspecific antibody binding, e.g., binding of Fc portion of antibody by the tissue.

Control Slide (Provided): Supplied control slides, each contain 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 procedure. The evaluation of the DAKO-supplied control slide cell lines 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 tissue control for each set of test conditions should be included in each staining run.

The tissues used for the positive tissue controls should give weak positive staining so they can detect subtle changes in the primary antibody sensitivity. The control slides supplied with this system 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 human breast carcinoma tissue for the ideal positive tissue control.

Known positive tissue controls 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 tissue controls fail to demonstrate appropriate positive staining, results with the test 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. The variety of different cell types present in most tissue sections offers internal negative control sites (this should be verified by the user). **Colon, liver or thyroid** are appropriate for negative control tissue.

If specific staining occurs in the negative control tissue, results with the patient specimens should be considered invalid.

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

Assay Verification: Prior to initial use of an antibody or staining system in a diagnostic procedure, the user should verify the antibody's specificity by testing it on a series of in-house tissues with known immunohistochemical 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 NCCLS Quality Assurance for Immunocytochemistry, Proposed Guideline.[23] These quality control procedures should be repeated for each new antibody lot, or whenever there is a change in assay parameters. Breast carcinomas with known HER2 protein staining intensities from 0-3+ and negative tissues, e.g., colon, liver or thyroid are suitable for assay verification.

INTERPRETATION OF STAINING

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. 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 1+, 2+ and 3+ staining, refer to the DAKO HercepTest™ brochure for representative pictures of the staining intensities.

Table 2
Cell Membrane Staining Intensity Criteria

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

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

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

Table 3
Order of Slide Evaluation

Slide Reading Order	Rationale
1. Control slide containing the three cell lines	Presence of 3+ brown cell membrane staining (rimming) in the control cell line SKBR3, partial brown rimming in the 1+ control cell line MDA-175, and no staining in the control cell line MDA-231 (0) indicates a valid assay. Presence of brown staining in the 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	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	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.
4. Patient tissue stained using the negative reagent control	Absence of specific staining verifies the specific labeling of the target antigen by the primary antibody. Other tan or brown staining occurring in the cytoplasm of the specimen treated with the Negative Control Reagent such as in connective tissue, leucocytes, erythrocytes, or necrotic tissue should be considered nonspecific background staining and should be reported under the comments section of the data spreadsheet.
5. Patient tissue 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.

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

Presence of circumferental brown cell membrane staining (rimming) in the control cell line SKBR3, partial brown rimming in the control cell line MDA-175, and no staining in the 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 test specimens should be considered invalid.

Positive Control Tissue: The positive tissue control 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 nonspecifically.[25] Staining should be observed in tumor tissue as brown, cell membrane staining. Brown staining of the cytoplasm and negative tissues within the specimen should be no more than 1+ staining intensity score.

Negative Control Tissue: The negative control tissue should be examined after the positive control tissue to verify the specificity of the labeling of the target antigen by the primary antibody. The absence of specific staining in the negative control tissue confirms the lack of kit cross-reactivity to cells/cellular components. If specific staining occurs in the negative control tissue, results with the patient specimen should be considered invalid. Alternatively, negative portions of the positive control tissue may serve as the negative control tissue, but this should be verified by the user. Note that a weak reaction (0-1+ staining intensity) can be observed in most normal epithelial tissue. Possible negative control tissues include: colon,liver or thyroid.

Nonspecific 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.

Patient Tissue: Examine patient specimens stained with HercepTest™ last. Positive staining intensity should be assessed within the context of any nonspecific background staining of the negative control reagent. As with any immunohistochemical 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.

General Limitations

1. Immunohistochemistry is a multistep diagnostic process that requires specialized training in the selection of the appropriate reagents; tissue selection, fixation, and processing; preparation of the IHC 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 nonspecific staining with horseradish peroxidase.[26]

6. Reagents may demonstrate unexpected reactions in previously untested tissues. The possibility of unexpected reactions even in tested tissue groups cannot be completely eliminated due to biological variability of antigen expression in neoplasms, or other pathological tissues.[27] Contact DAKO Technical Services with documented unexpected reaction.

7. False-positive results may be seen due to non-immunological binding of proteins or substrate reaction products. They may also be caused by pseudoperoxidase activity (erythrocytes), endogenous peroxidase activity (cytochrome C).[27]

Product Specific Limitations

1. The antigen present in the control cell line, MDA-175 is subject to degradation over time. Assess the control slide results in connection with the date of manufacture of the cell line slide. Negative staining of the MDA-175 cells may only indicate that the control slide has degraded.

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).[28]

3. For optimal and reproducible results, the HER2 protein requires heat-induced epitope retrieval when tissues are routinely fixed (neutral buffered formalin or Bouin's) and paraffin embedded. This pretreatment needs to be completed at the

beginning of the entire staining process. See the "Specimen Preparation Section, Treatment of Tissues Prior to Staining" for instructions.

4. Heat-induced epitope retrieval of the HER2 protein should only be done using a calibrated waterbath. Other methods of heating have been tested and do not give reproducible results.
5. Do not substitute reagents from other lot numbers of this kit, or from kits of other manufacturers.
6. False results could be obtained from evaluation of cytoplasmic staining. Consider only the intensity of cell membrane staining when interpreting results.

Performance Characteristics

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 DAKO HercepTest™ was developed to provide a comparable alternative to the CTA.

The safety and effectiveness of HERCEPTIN® was evaluated in a randomized controlled clinical trial and a large, open-labeled trial (See HERCEPTIN® package insert). All patients selected for the HERCEPTIN® clinical trials demonstrated overexpression of HER2 protein by immunohistochemistry testing performed with a clinical trial assay (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 suggest 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 size of the treatment effect. Because none of the patients in the HERCEPTIN® studies were selected using the DAKO 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 DAKO HercepTest™.

1) Comparison to the Clinical Trial Assay (CTA)

The DAKO HercepTest™ System 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. Figure 1 shows the results in a 2 X 2 diagram where 0 and 1+ were considered to be negative and 2+ and 3+ were positive.

Figure 1

A 2 x 2 Concordance of the DAKO HercepTest™ to the Clinical Trial Assay
(Number of specimens)

		Clinical Trial Assay		
		positive	negative	Total
DAKO-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 DAKO HercepTest™ to the CTA was 79% (431/548), with a 2-sided 95% confidence interval of 76%-82%. Twenty one percent (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 reactive (3+ staining intensity).

Figure 2

A 3 x 3 Concordance for DAKO HercepTest™ and Clinical Trial Assay

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

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

Accuracy

DAKO HercepTest™ was also tested on 2 microscope slides containing paraffin-embedded tissues 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 IHC on frozen tissues.[28] The results are presented in Figure 3.

Figure 3

Comparison of DAKO HercepTest™ to combined results (OE) from Gene Amplification and Overexpression tests.

		Reference OE Classification		
		+	–	Total
DAKO	+	43	0	43
HercepTest™	–	26	99	125
Kit	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 DAKO HercepTest kit and some degree of overexpression by the reference HER2 OE classification.

Reproducibility:

Intra-Run Reproducibility

Intra-run reproducibility was tested in one laboratory with 5 specimens of different IHC intensity staining scores. Each specimen was run in triplicate in a masked randomized format. This protocol was used with manual staining and again 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 IHC intensity staining scores randomized and masked using automated methodology. One laboratory also repeated this protocol using manual methodology. Excellent reproducibility was seen for positive versus negative results (0 and 1+ vs. 2+ and 3+) with the exception of two samples in one laboratory using the automated methodology 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 IHC staining intensity scores. Freshly cut slides were forwarded to each testing laboratory for manual and automated staining and evaluation by a pathologist. Inter-laboratory percent agreement ranged from 82% to 90% for a dichotomous positive/negative determination where 0 and 1+ were negative and 2+ and 3+ were positive for HER2 overexpression. This range included both manual and automated staining methods. 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 4 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 4
Summary of HercepTest™ Normal Tissue Reactivity

TISSUE TYPE (# tested)	POSITIVE TISSUE ELEMENT STAINING AND STAINING PATTERN

Adrenal (3)	None
Bone Marrow (3)	None
Brain/Cerebellum (3)	None
Brain/Cerebrum (3)	None
Breast (3)	Mammary gland (1+ staining intensity)
Cervix uteri (3)	None
Colon (3)	Columnar epithelium, surface (1+ staining intensity)
Esophagus (3)	Squamous epithelia (1/3 tissues, 2+ staining intensity)
Heart (3)	None
Kidney (3)	Tubule (1+ staining intensity)
Liver (3)	None
Lung (3)	None
Mesothelial Cells (3)	None
Ovary (3)	None
Pancreas (3)	Langerhans cells, Cytoplasmic (3+ staining intensity)
Parathyroid (3)	None
Peripheral Nerve (3)	None
Pituitary (3)	Endocrine cells (Cytoplasmic, 3+ staining intensity)
Prostate (3)	Prostate gland (2+ staining intensity)
Salivary Gland (3)	None
Skeletal Muscle (3)	None
Skin (3)	None
Small Intestine (3)	Columnar epithelium, surface (1+ staining intensity)
Spleen (3)	None
Stomach (3)	Epithelium (1/3 tissues, 1+ staining intensity)
Testis (3)	None
Thymus (3)	None
Thyroid (3)	None
Tonsil (3)	Squamous epithelia (2+ staining intensity)
Uterus (3)	Endometrium (1/3 tissues, 1+ staining intensity)

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

TROUBLESHOOTING

Refer to the Troubleshooting section in the previously referenced handbook[18] for remedial action, or contact DAKO's Technical Service Department to report unusual staining.

PROBLEM	PROBABLE CAUSE	SUGGESTED ACTION
1. No staining of any slides.	1a. Reagents not used in proper order. 1b. Sodium azide in buffer bath.	1a. Review application of reagents. 1b. Use fresh, azide-free buffer.
2. Weak staining of all slides.	2a. Sections retain too much solution after wash bath. 2b. Slides not incubated long enough with antibodies or substrate-chromogen.	2a. Gently tap off excess solution before wiping around section. 2b. Review recommended incubation times.
3. Excessive background staining in all slides.	3a. Specimens contain high endogenous peroxidase activity. 3b. Paraffin incompletely removed. 3c. Slides not properly rinsed. 3d. Faster than normal substrate-chromogen reaction due to excessive room temperature. 3e. Sections dried during staining procedure. 3f. Nonspecific binding of reagents to tissue section.	3a. Use longer incubation time of Peroxidase-Blocking Reagent, Vial No. 1. 3b. Use fresh xylene or toluene baths. If several slides are stained simultaneously, the second xylene bath should contain fresh xylene. 3c. Use fresh solutions in buffer baths and wash bottles. 3d. Use shorter incubation time with substrate-chromogen solution. 3e. Use humidity chamber. Wipe only three to four slides at a time before applying reagent. 3f. Check fixation of the specimen and the presence of necrosis.

4. Tissue detaches from slide	4a. Use of incorrect slides	4a. Use SuperFrost Plus, poly-L-lysine coated or DAKO Silanized Slides.
5. Excessive strong specific staining	5a. Incubation with primary antibody or Visualization Reagent too long.	5a. Review recommended incubation times.
6. Lack of staining of the 1+ control slide cell line	6a. Inadequate heat induced epitope retrieval 6b. Lack of reaction with the Substrate-Chromogen Solution (DAB) 6c. Degradation of control slide	6a. Verify the waterbath temperature for a full 40 minutes at a minimum of 95°C. 6b. Use a full 10 minutes incubation of the Substrate-Chromogen Solution. 6c. Check expiration date and kit storage conditions.

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 the DAKO Technical Services for further assistance.

Additional information on staining techniques and specimen preparation can be found in the Handbook: Immunochemical Staining Methods[18] (available from DAKO), Atlas of Immunohistology[29] and Immunoperoxidase Techniques, A Practical Approach to Tumor Diagnosis.[30]

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