

## B. PACKAGE INSERT

Catalog No.: RD134-60K and RD134-YCX

**InSite™ Her-2/neu**

Lot No:      xxxx

### I. Intended Use: For *In Vitro* Diagnostic Use:

InSite™ Her-2/neu Mouse Monoclonal Antibody (Clone CB11) kit is intended for *In Vitro* Diagnostic use in Immunohistochemistry (IHC) assays to semi-quantitatively localize by light microscopy the over-expression of Her-2/neu (i.e., c-erbB-2) in formalin-fixed, paraffin-embedded normal and neoplastic tissue sections. InSite™ Her-2/neu is indicated as an aid in the assessment of breast cancer patients for whom Herceptin® (Trastuzumab) therapy is being considered. Clinical interpretation of InSite™ Her-2/neu immunostaining results (absence or presence; semi-quantitative intensity score) should be complemented by appropriate controls and morphological tissue analysis and be evaluated by a qualified pathologist within the context of patient clinical history and other diagnostic results.

(Note: 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 InSite™ Her-2/neu. The InSite™ Her-2/neu was compared to the HercepTest® on an independent sample and found to provide acceptably concordant results. The actual correlation of InSite™ Her-2/neu to clinical outcome has not been established.)

| <u>Catalog No.</u> | <u>Description</u>   |
|--------------------|--|
| RD134-60K          | InSite™ Her-2/neu (For Manual Immunohistochemical Staining.)<br>(The kit is for 60 tests.)     |
| RD134-YCX          | InSite™ Her-2/neu (For Automated Immunohistochemical Staining.)<br>(The kit is for 200 tests.) |

### II. Summary and Explanation:

The InSite™ Her-2/neu Monoclonal Antibody (Clone: CB11) was raised against an internal domain of the Her-2 (c-erbB-2 oncoprotein) receptor, a 185kDA transmembrane glycoprotein with tyrosine kinase activity.<sup>1, 10</sup> This antibody identifies a protein of ~190 kDA in Western Blot analysis of crude cell protein

extract from breast cancer tumor cell lines SK-BR3 and AU565. The Her-2 gene was first identified in 1981 as a transforming oncogene in 3-methylcholanthrene-induced rat neural tumors.<sup>26</sup> Subsequent analysis of the 185 kDA protein revealed this protein to be a member of the epidermal growth factor receptor (EGFR) family that includes EGFR (Her-1, erbB-1), Her-3 (erbB-3) and Her-4 (erbB-4).<sup>1</sup> The Her-2/neu receptor is unique in the sense that, to date, no directly binding ligand to this receptor has been isolated but it is the favored heterodimerization partner of each ligand bound family member.<sup>3, 13, 24, 33</sup> Approximately 20-30% of breast cancer cases present gene amplification and/or protein over-expression of the Her-2 receptor.<sup>14, 28, 35, 36</sup> About 15-30% of cases of invasive ductal cancers, 50-90% of ductal carcinoma *in situ*, and almost all cases of Paget's disease were found to exhibit upregulated Her-2 protein expression by multiple researchers.<sup>12, 15, 17</sup> Since the availability of therapy (Trastuzumab) directed toward the Her-2 receptor, laboratory testing of Her-2 expression in breast carcinoma has become very important in patient care.

### III. Principle of Procedure:

InSite™ Her-2/neu (Clone: CB11) may be used to detect Her-2 receptor antigen in formalin-fixed, paraffin-embedded tissue sections after pretreatment with Citra Plus Antigen Retrieval. This pretreatment is essential for optimal reactivity of InSite™ Her-2/neu with Her-2 receptor antigen in routinely processed surgical pathology specimens. In general, Immunohistochemical staining techniques allow for the visualization of antigens via the sequential application of a specific antibody to the antigen (primary antibody), a secondary antibody to the primary antibody (link antibody), an enzyme complex and a chromogenic substrate interspersed with washing steps. The enzymatic activation of the chromogen results in a visible reaction product at the antigen site. Finally, the specimen may be counterstained and mounted/cover slipped. Results are interpreted using light microscopy and aid in the differential diagnosis of pathophysiological processes, which may or may not be associated with a particular antigen.

InSite™ Her-2/neu may be used in manual Immunohistochemical applications or in automated applications, such as on the BioGenex i6000™ Automated Staining System or the OptiMax® Plus Consolidated Staining System. The latter Automated Staining Systems will perform all the staining steps as described in their respective Operator's Manual. After completion of the staining procedure, the stained slides are removed from the instrument and mounted manually with an appropriate mounting medium.

### IV. Reagents Provided

#### A. Primary Antibody: InSite™ Her-2/neu

(Provided in 6 ml or 20 ml volumes for 60 or 200 tests, respectively.)

- Immunogen: Synthetic peptide corresponding to a site on the internal domain of the Her-2 (c-erbB-2) protein
- Clone: CB11<sup>10</sup>

- Immunoglobulin class: Mouse monoclonal IgG1
- Total protein concentration: 10-15 mg/ml
- Specificity: Human Her-2 receptor (c-erbB2 oncoprotein).
- Method: Tissue culture supernatant diluted in PBS containing BSA carrier protein and 0.09% sodium azide.
- Specific IgG concentration and percent of total protein available on request.

## B. Materials Provided

InSite™ Her-2/neu antibody has been optimally diluted for use in either manual IHC or automated IHC applications performed on BioGenex Automated Staining Systems and should not require further dilution. Further dilution may result in loss of antigen staining. The user must validate any such change.<sup>34</sup> Differences in tissue processing and technical procedure in the end user's laboratory may produce significant variability in results, necessitating regular performance of in-house controls (see Quality Control section).

| InSite™ Her-2/neu (Cat. No. RD134-60K) |   | For 60 Tests |              |
|--|---|--------------|--------------|
| Item Number                            | Description                                     | Volume       | No. of Vials |
| HK083-5K                               | Power Block                                     | 6 ml         | 1            |
| HK111-5K                               | Peroxide Block                                  | 6 ml         | 1            |
| HK330-5K                               | HRP Label                                       | 6 ml         | 1            |
| HK340-5K                               | MultiLink®                                      | 6 ml         | 1            |
| HK128-5K                               | DAB Substrate Buffer                            | 2.5 ml       | 6            |
| HK124-5K                               | Liquid DAB Chromogen                            | 2 ml         | 1            |
| HK126-5K                               | DAB Substrate                                   | 2 ml         | 1            |
| HK119-5M                               | Negative Reagent Control                        | 3 ml         | 1            |
| AM134-5MP                              | InSite™ Her-2/neu Antibody                      | 6 ml         | 1            |
| HK080-5K                               | Citra Plus (10X Concentrated)                   | 100 ml       | 1            |
| HK583-YAK                              | Super Sensitive™ Wash Buffer (20X Concentrated) | 100 ml       | 1            |
| CL134MT-5                              | Positive and Negative Control Slide*            | 5 slides     | 1 pack       |
| HK100-5K                               | Hematoxylin                                     | 6 ml         | 1            |

| InSite™ Her-2/neu (Cat. No. RD134-YCX) |                                      | For 200 Tests |              |
|--|--------------------------------------|---------------|--------------|
| Item Number                            | Description                          | Volume        | No. of Vials |
| HK083-20K                              | Power Block                          | 20 ml         | 1            |
| HK111-20K                              | Peroxide Block                       | 20 ml         | 1            |
| HK330-20K                              | HRP Label                            | 20 ml         | 1            |
| HK340-20K                              | MultiLink®                           | 20 ml         | 1            |
| HK128-20X                              | DAB Substrate Buffer                 | 20 ml         | 5            |
| HK124-7K                               | Liquid DAB Chromogen                 | 4 ml          | 1            |
| HK126-7K                               | DAB Substrate                        | 3 ml          | 1            |
| HK119-10X                              | Negative Reagent Control             | 10 ml         | 1            |
| AM134-20MP                             | InSite™ Her-2/neu Antibody           | 20 ml         | 1            |
| HK080-5K                               | Citra Plus (10X Concentrated)        | 100 ml        | 2            |
| CL134MT-5                              | Positive and Negative Control Slide* | 5 slides      | 1 pack       |
| HK100-20K                              | Hematoxylin                          | 20 ml         | 1            |

\* Positive and Negative Control Slide: Each slide contains sections of formalin-fixed, paraffin-embedded breast carcinomas from cell lines representing different levels of Her-2 expression.

## V. Materials Required But Not Provided:

### A. Additional Reagents Not Provided

- EZ-DeWax™ (BioGenex; Cat. No. HK585-5K)
- Xylene
- Ethanol (Absolute and 95%)
- Distilled or deionized water
- Ammonia water (1% ammonium hydroxide)
  - Permanent: For use with DAB substrate (Fisher; Cat. No. SP15-100)
- 10% Buffered Formalin (for specimen preparation)
- Super Sensitive™ Wash Buffer (20X) (BioGenex; Cat. No. HK583-YAK) (N.B. For use with InSite™ Her-2/neu, Cat. No. RD134-YCX only.)
- Mounting medium (Aqueous Mounting Media; BioGenex; Cat. No. HK099-5K)

### B. Laboratory Equipment Not Provided

- Oven or incubator (capable of maintaining 56-60°C)
- BioGenex Automated Staining System
- Humidity Chamber
- Microwave oven
- Staining Jars or baths
- Timer (capable of 3-20 minute intervals)
- Wash Bottles
- Absorbent Wipes
- Microscopes slides (pre-treated with poly-L-Lysine) (BioGenex; Cat. No. XT002-SL)
- Coverslips (VWR; Cat. No. 48366-089)
- Lens paper
- Light microscope with magnification of 200X

## VI. Storage:

InSite™ Her-2/neu components should be stored at 2-8°C (36-46°F). Do not freeze.

InSite™ Her-2/neu is suitable for use up to six months from the point of manufacture when stored at 2-8°C. Do not use the product after the expiration date printed on the vial. If reagents are stored under any conditions other than those specified in the package insert, they must be verified by the user.<sup>34</sup>

Positive and negative controls should be run simultaneously with all patient specimens. If unexpected staining is observed which cannot be explained by variations in laboratory procedures and a problem with the kit is suspected, please immediately contact BioGenex Technical Support at Tel. No. (800) 421-4149.

## VII. Specimen Preparation:

10% formalin-fixed and paraffin embedded tissues should be collected on positively charged slides (BioGenex; Cat. No. XT002-SL). Tissues fixed in 10% (v/v) formalin are suitable for use prior to paraffin embedding. Consult references for further details on specimen preparation.<sup>16,25</sup>

Properly fixed and embedded tissues expressing the Her-2 receptor antigen should be stored in a cool place. The Clinical Laboratory Improvement Act (CLIA) 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."<sup>34</sup>

## VIII. Treatment of Tissues Prior to Staining:

Slides containing formalin-fixed, paraffin-embedded tissue sections should be stored in a dry place at room temperature (20-26°C). After deparaffinization (BioGenex EZ-DeWax™; Cat. No. HK585-5K& HK584-5K), tissue sections must be treated with BioGenex Citra Plus Antigen Retrieval Solution (Cat. No. HK080-5K) using microwave irradiation before use with InSite™ Her-2/neu. InSite™ Her-2/neu antibody may not produce an optimal signal in paraffin sections where the antigen retrieval pretreatment procedure is omitted.

## IX. Precautions:

- (1) This product is intended for *In Vitro* Diagnostic use.
- (2) This product contains no hazardous material at a reportable concentration according to U.S. 29 CFR 1910.1200, OSHA Hazard Communication Standard and EC Directive 91/155/EC. Some product(s) or component(s) of the kit may contain sodium azide, if indicated below, at concentrations of less than 0.1%. Sodium azide is not classified as a hazardous chemical at these product concentrations. However, toxicity information regarding sodium azide at these product concentrations has not been thoroughly investigated. For more information, a Material Safety Data Sheet for sodium azide in pure form is available upon request.
- (3) Sodium azide ( $\text{NaN}_3$ ; 0.09%), used as a preservative in InSite™ Her-2/neu, is toxic if ingested. Sodium azide may react with lead or copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide build-up in plumbing.<sup>7, 19</sup> Sodium azide may be fatal if inhaled, swallowed, or absorbed through the skin. In case of exposure, obtain medical attention immediately.
- (4) Organic reagents (e.g., ethanol and xylene) are flammable. Do not use near any open flame. Methanol is a poison; do not ingest.

- (5) Formaldehyde, 37% solution (formalin), used in specimen preparation, is harmful if inhaled, swallowed, or absorbed through the skin. It is classified as a potential carcinogen and may alter genetic material. Avoid inhalation, ingestion, or contact with the skin. If contacted with eyes or skin, flush immediately with copious amounts of cold water. Formalin is also combustible and should be used away from any open flame.
- (6) DAB, and xylene are classified as possible carcinogens and can cause skin irritation upon contact. Avoid contact with skin. If skin contact should occur, flush immediately with copious amounts of water.
- (7) DAB chromogen may be affected if exposed to excessive light levels, such as direct sunlight. Do not perform staining in strong light.
- (8) Hydrogen peroxide is a corrosive and a strong oxidizer. Contact with other materials may cause a fire.
- (9) Refer to appropriate product inserts for *instructions of use* and *safety information* (i.e., Material Safety Data Sheet) on detection reagents and other materials that may be used with the InSite™ Her-2/neu monoclonal antibody.
- (10) Consult Federal, State and local regulations for disposal of any potentially toxic components.
- (11) 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.<sup>34</sup>
- (12) Do not pipette reagents by mouth, and avoid contact of reagents and specimens with skin and mucous membranes. If reagents or specimens come in contact with any sensitive areas, wash with copious amounts of water.
- (13) Minimize microbial contamination of reagents or increase in nonspecific staining may occur.
- (14) Incubation times or temperatures other than those specified may give erroneous results. The user must validate any such change.<sup>34</sup>

**X. Instruction for Use:**

**Note: If using any BioGenex Automated Staining Systems, please refer to the respective Operator's Manual for guidance on instrument operation.**

| Parameters   | BioGenex Recommendations      |
|--------------|-------------------------------|
| Tissue Type  | Paraffin-embedded             |
| Pretreatment | Citra Plus™ Antigen Retrieval |

|                                 |                             |
|---------------------------------|-----------------------------|
| Control Tissue                  | Breast Carcinoma            |
| Incubation Time and Temperature | Room Temperature (20–26° C) |

**A. Reagent Preparation**

- **Citra Plus Antigen Retrieval:** Prepare a sufficient volume of Citra Plus from the 10X stock solution provided with this kit. Dilute concentrated Citra Plus 1:10 using distilled water (i.e., 1 part plus 9 parts of distilled water). Unused concentrated and diluted Citra Plus may be stored at 4-8° C for 1 month. Both concentrated and diluted Citra Plus should be clear in appearance. Do not use if either concentrated or diluted solution appears cloudy.
- **Super Sensitive™ Wash Buffer:** Prepare a sufficient volume of Super Sensitive™ Wash Buffer from the 20X stock solution. Dilute concentrated Super Sensitive™ Wash Buffer 1:20 using distilled water (i.e., 1 part plus 19 parts of distilled water). Unused concentrated and diluted Super Sensitive™ Wash Buffer may be stored at 4-8° C for 1 month. Both concentrated and diluted Super Sensitive™ Wash Buffer should be clear in appearance. Do not use if either concentrated or diluted solution appears cloudy.
- **Substrate-Chromogen Working Solution:** This solution must be prepared before use.

Mix the DAB chromogen solution thoroughly before using.

**Manual Kit:** Add 2 drops of well-mixed Liquid DAB chromogen (HK124-5K) and 1 drop of DAB Substrate (HK126-5K) to one bottle of 2.5 ml ready-to-use DAB Substrate Buffer (HK128-5K).

**Automation Kit:** Add 16 drops of well-mixed Liquid DAB chromogen (HK124-7K) and 8 drops of DAB Substrate (HK126-7K) to one bottle of 20 ml ready-to-use DAB Substrate Buffer (HK128-20X).

With cap in place, mix the working solution thoroughly.

Each 2.5 ml of working solution is sufficient to stain up to 25 slides.

This solution is stable for 6 hours when stored at room temperature (20–26° C).

- **Mounting Media:** Use of either aqueous or permanent non-aqueous mounting media is appropriate for slides stained with InSite™ Her-2/neu. BioGenex recommends Aqueous Mounting Media (BioGenex; Cat. No.: HK099-5K) for use with InSite™ Her-2/neu.

## B. Staining Procedure

**These instructions should be reviewed in full before use of InSite™ Her-2/neu.**

Never let tissue specimens become dehydrated during the following staining procedure. Tissue sections that become dehydrated at any point during the staining procedure may exhibit increased non-specific background staining. Always keep tissue sections sufficiently covered with reagent, Super Sensitive™ Wash Buffer or distilled water. This staining procedure may be delayed by up to one hour if tissue sections are kept sufficiently covered with Super Sensitive™ Wash Buffer or distilled water.

### **Step 1: Deparaffinization and Rehydration**

Before staining, all tissue sections must be deparaffinized and rehydrated to remove all embedding medium. Failure to remove all paraffin from tissue sections prior to staining may result in increased non-specific staining. Traditional xylene based deparaffinization methodologies are appropriate for use with InSite™ Her-2/neu. BioGenex recommends the use of EZ-DeWax™ (BioGenex; Cat. No. HK585-5K), a non-toxic, easy, one-step reagent solution for deparaffinization and rehydration of slide bound tissue sections.

### **Step 2: Citra Plus Antigen Retrieval**

Following rehydration of tissue sections, immerse slides in 1X Citra Plus Antigen Retrieval Solution in a microwave-safe vessel (e.g., Coplin jars, NordicWare® Microwave Tender Cooker). Turn the microwave oven on high power (500 – 1,000 watts) and closely watch the solution until it comes to a rapid boil, and then turn off the oven. (Note: It usually takes 3-7 minutes before a boiling point is reached. However, the amount of time required may vary significantly depending on a number of factors, such as the temperature of the Antigen Retrieval solution, the wattage of the microwave oven, the age of the oven and the inside temperature of the oven. It is very important that a rapid boil is reached for every run before proceeding to the next step.) Set oven power to approximately 20-50% level and heat for 10 to 15 minutes. (Note: The power setting should be adjusted so that the oven cycles on and off every 20-30 seconds and the solution boils about 5-10 seconds each cycle.) The reuse of Citra Plus Antigen Retrieval Solution is not recommended. Discard the used Antigen Retrieval Solution as a chemical waste.

**Step 3:** Apply 2-3 drops of **Peroxide Block** or enough amount to cover the entire tissue section for 10 minutes at room temperature. Rinse with Super Sensitive™ Wash Buffer. Gently tap off excess buffer.

**Step 4:** Apply 2-3 drops of **Power Block** or enough amount to cover the entire tissue section for 10 minutes at room temperature. Rinse with Super Sensitive™ Wash Buffer. Gently tap off excess buffer.

**Step 5:** Apply 2-3 drops of **InSite™ Her-2/neu Antibody or Negative Reagent Control** or enough amount to cover the entire tissue section. Incubate at room temperature for 30 minutes. Rinse with Super Sensitive™ Wash Buffer. Gently tap off excess buffer.

**Step 6:** Apply 2-3 drops of **MultiLink®** or enough amount to cover the entire tissue section. Incubate at room temperature for 20 minutes. Rinse with Super Sensitive™ Wash Buffer. Gently tap off excess buffer.

**Step 7:** Apply 2-3 drops of **HRP Label** or enough amount to cover the entire tissue section. Incubate at room temperature for 20 minutes. Rinse with Super Sensitive™ Wash Buffer. Gently tap off excess buffer.

**Step 8:** Apply 2-3 drops of **Substrate-Chromogen** or enough amount to cover the entire tissue section. Incubate at room temperature for 10 minutes. Reaction is complete when a reddish- brown precipitate becomes visible in tissue section. Rinse with Super Sensitive™ Wash Buffer. Gently tap off excess buffer.

**Step 9:** Apply 2-3 drops of **(Mayer's) Hematoxylin** or enough amount to cover the entire tissue section. Allow stain reaction to occur at room temperature for 1 minute. Rinse with Super Sensitive™ Wash Buffer. Gently tap off excess buffer. Depending on the incubation length and potency of hematoxylin used, counter staining will result in a pale to dark blue coloration of the cell nuclei.

**Step 10: Mounting**

Use of either aqueous or permanent non-aqueous mounting media is appropriate for tissues stained with InSite™ Her-2/neu. BioGenex recommends Aqueous Mounting Media (BioGenex; Cat. No. HK099-5K) for use with InSite™ Her-2/neu. Store slides in the dark at room temperature.

**If using any BioGenex Automated Staining Systems, please refer to the respective Operator's Manual for guidance on instrument operation.**

The stained slides are stable for at least 2 years, if stored in a dry place at room temperature (20-26°C), after completion of the staining and mounting procedures as described in this product insert.

## **XI. Quality Control:**

Differences in tissue processing and technical procedures in the end user's laboratory may produce significant variability in results, necessitating regular performance of in-house controls. The following controls should be used during the staining procedure.

### **A. Positive Tissue Control**

An external positive control from human breast carcinoma should be fresh autopsy/biopsy/surgical specimens, fixed, processed and embedded as soon as possible in the same manner as patient sample(s). Positive tissue controls are indicative of correctly prepared tissues and proper staining techniques. Such a control monitors all steps of the analysis, from tissue preparation through staining. Use of a tissue section fixed or processed differently from the test specimen will control for all reagents and method steps, except fixation and tissue processing. One external positive tissue control for each set of test conditions should be included in each staining run.

The tissues used for the external positive control materials should be selected from patient specimens with well-characterized, low levels of the positive target antigen that give weak positive staining. The low level of positivity for external positive controls is designed so to ensure detection of subtle changes in primary antibody sensitivity from instability or problems with the IHC methodology. BioGenex Positive Control Slide for Her-2/neu (Cat. No. CL134MT-5) or other specimens processed differently from the patient sample(s) can be used as a positive control to validate reagent performance only. They cannot verify tissue preparation. In the case of InSite™ Her-2/neu monoclonal antibody, use of a known 2+ breast carcinoma is recommended when prepared in the manner specified above.

Known positive tissue controls should only be utilized for monitoring the correct performance of processed tissues and test reagents, rather than as an aid in formulating a specific diagnosis of patient samples. If the positive tissue controls fail to demonstrate positive staining, results with the test specimens should be considered invalid.

### **B. Negative Tissue Control**

Cytoplasmic staining is seen in certain non-neoplastic tissues. Hence, a negative control tissue should be selected with proper care. Use a negative tissue control, fixed, processed and embedded in a manner identical to the patient sample(s) with each staining run to verify the specificity of the IHC primary antibody for demonstration of the target antigen, and to provide an indication of specific background staining (false positive staining). Alternatively, the variety of different cell types present in most tissue sections can offer internal negative control sites to verify the assay performance specifications, but this practice must be verified by the user. Heart, lung or cervix are recommended as negative control tissue. Refer to

Performance Characteristics section of this package insert for a list of the type and sources of specimens that may be used for negative tissue controls.

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

**C. Non-Specific Negative Reagent Control**

An affinity purified normal mouse IgG is provided with this kit and should be used as a negative control reagent in place of InSite™ Her-2/neu antibody with one section of each patient specimen to evaluate non-specific staining and allow better interpretation of specific staining at the antigenic site for InSite™ Her-2/neu. The incubation time of the negative reagent control should be the same as InSite™ Her-2/neu antibody.

When panels of several antibodies are used on serial sections, the negatively staining areas of one slide may serve as negative/nonspecific binding background controls for other antibodies.

To differentiate endogenous enzyme activity or nonspecific binding of enzymes from specific immunoreactivity, additional patient tissues may be stained exclusively with substrate-chromogen or enzyme complexes (streptavidin) and substrate-chromogen, respectively.

**XII. Assay Verification:**

Prior to initial use of InSite™ Her-2/neu in a diagnostic/prognostic procedure, the end user should verify InSite™ Her-2/neu specificity by testing it on a series of in-house tissues with known IHC performance characteristics representing positive and negative Her-2/neu immunostaining scores. Refer to the Quality Control procedures previously outlined in this product insert and to the quality control recommendations of the College of American Pathologists (CAP) Certification Program for Immunohistochemistry and/or the NCCLS IHC guideline.<sup>9, 21</sup> These quality control procedures should be repeated for each new InSite™ Her-2/neu lot, or whenever there is a change in assay parameters. Tissues listed in the Performance Characteristics section are suitable for assay verification.

**XIII. Interpretation of Staining:**

**A. Positive Control Slides**

Positive Control slides provided in the kit should be stained and examined first to ascertain assay validity. Three cell lines used on control slides are, SK-BR3 (3+ over-expression), MDA-MB-175 (1+ expression), and MDA-MB-231 (no expression). A brown circumferential cell membrane staining of the SK-BR3 cells is considered as 3+, and MDA-MB-175 cells show discontinuous membrane staining in few cells on the membrane and occasional cytoplasmic staining. This is considered as a 1+. MDA-MB-

231 shows no cell membrane staining. If any pattern is observed outside these scoring criteria, all results with the patient specimens shall be considered invalid. This slide should not be used as a scoring guide.

**B. Positive Tissue Control**

The positive tissue control stained with InSite™ Her-2/neu should be examined next to confirm that proper antigen retrieval method and fixation of sample are used. The presence of a reddish-brown (3,3' diaminobenzidine tetrachloride, DAB) reaction product on the tumor cell membrane is indicative of positive reactivity. Cytoplasmic staining in the absence of membrane staining should be considered as negative. If the positive tissue control fails to demonstrate proper positive staining in the cell membrane, any results with the test specimens should be considered invalid.

The color of the reaction product may vary if substrate chromogens other than those stated are used. Depending on the incubation length and potency of hematoxylin used, counter staining will result in a pale to dark blue coloration of the cell nuclei.

**C. Negative Tissue Control**

The negative tissue control stained with InSite™ Her-2/neu should be examined after the positive tissue control to verify the specificity of InSite™ Her-2/neu for the membrane bound Her-2 receptor. The absence of specific staining in the negative tissue control confirms the lack of antibody cross-reactivity to cells/cellular components. No positive staining (i.e., Intensity Scores of 1+, 2+, or 3+; see Table 1 below) should be observed in nuclei, cytoplasm and membrane of cells in the negative control tissues, e.g., lung. If specific staining (false positive staining) occurs in the negative external tissue control, results with the patient specimen should be considered invalid.

Nonspecific staining, if present, usually has a diffuse appearance. Sporadic staining of connective tissue may also be observed in sections from excessively formalin-fixed tissues. Necrotic or degenerated cells often stain nonspecifically.<sup>18</sup> Use intact cells for interpretation of staining results.

**D. Patient Tissue**

Examine patient specimens stained with InSite™ Her-2/neu last. Positive staining intensity should be assessed within the context of any nonspecific background staining of the negative reagent control. For the determination of Her-2/neu protein expression, only the membrane staining intensity and pattern should be evaluated. Cytoplasmic staining should be considered non-specific staining and should not be included in the assessment of membrane staining intensity. (The antibody produced by Clone CB11 occasionally shows cytoplasmic staining and presence of cytoplasmic

staining alone in the absence of membrane staining should be considered as negative.)

The following scoring criteria should be used when assessing the intensity of cell membrane staining with InSite™ Her-2/neu.

**Table 1. Criteria for Membrane Staining Intensity.**

| Staining pattern  | Score         |
|---|---------------|
| No staining or membrane staining in fewer than 10% of tumor cells   | 0 (negative)  |
| Faint, barely perceptible membrane staining in more than 10% of tumor cells, the cells are stained only in part of the membrane | 1+ (negative) |
| Weak to moderate complete membrane staining observed in more than 10% of tumor cells  | 2+ (positive) |
| Strong, complete membrane staining in more than 10% of tumor cells  | 3+ (positive) |

**Table 2: The Purpose of Daily Quality Control.**

| Tissue: Fixed & Processed Patient Sample   | Specific Antibody & Secondary Antibody  | Nonspecific Antibody* or Buffer plus Same Secondary Antibody as Used with Specific Antibody |
|--|---|---|
| <b>Positive Control:</b> Tissues or cells containing target antigen to be detected (could be located in patient tissue). <i>The ideal control is a weakly positive staining tissue to be most sensitive to antibody degradation.</i> | Controls all steps of the analysis. Validates reagent and procedures used for staining. | Detection of non-specific background staining.  |
| <b>Negative Control:</b> 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.  |
| <b>Patient Tissue</b>  | Detection of specific staining.   | Detection of non-specific background staining.  |

\* Same source and type 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.

Refer to the relevant sections in this package insert on Summary and Explanation, General Limitations, and Performance Characteristics for specific information regarding InSite™ Her-2/neu immunoreactivity.

## **XIV. General Limitations:**

### **A. General Limitations**

Immunohistochemistry 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 IHC slide and interpretation of the staining results.

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.<sup>18</sup> Excessive or incomplete counterstaining may compromise proper interpretation of results.

The clinical interpretation of any positive or negative staining result should be evaluated within the context of clinical presentation, morphology and other histopathological criteria. The clinical interpretation of any positive or negative staining result should be complemented by morphological studies using proper positive and negative internal and external controls as well as other diagnostic tests. Staining is to be performed in a certified licensed laboratory under the supervision of a qualified pathologist who is familiar with the proper use of IHC antibodies, reagents and methods to interpret the stained preparation. The pathologist is responsible for reviewing the stained slides and assuring the adequacy of positive and negative controls.

BioGenex manufactures InSite™ Her-2/neu at optimal dilution for use on prepared tissue sections following the instructions provided. Any deviation from recommended assay procedure may invalidate declared expected results; appropriate controls must be employed and documented. Users who deviate from recommended assay procedure must accept responsibility for interpretation of patient results under these circumstances.

Unexpected negative reactions in poorly differentiated neoplasms may be due to loss or marked decrease of expression of the antigen or loss of mutation(s) in the gene(s) coding for the antigen. Unexpected positive staining in tumors may be from expression of an antigen not usually expressed in morphologically similar normal cells, or from the persistence or acquisition of an antigen in a neoplasm that develops morphologic and immunological staining features associated with another cell lineage (divergent differentiation). Histopathological classification of tumors is not an exact science and some literature reports of unexpected staining may be controversial.

This product is not intended for use in flow cytometry. Performance characteristics have not been determined for flow cytometry.

Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit nonspecific staining with horseradish peroxidase.<sup>22</sup>

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 and other pathological tissues. Please contact BioGenex Technical Support at Tel. No. (800) 421-4149 with documented unexpected reaction(s).

False-positive results may be seen due to non-immunological binding of proteins or substrate reaction products. They may also be caused by pseudo-peroxidase activity (erythrocytes), endogenous peroxidase activity (cytochrome C), or endogenous biotin (e.g., liver, breast, brain, kidney) depending on the type of immunostain used.

#### **B. Product Specific Limitations**

Pretreatment of tissue sections by heating in Citra Plus Antigen Retrieval Solution (BioGenex Cat. No. HK080-5K) is required before staining with InSite™ Her-2/neu. An end-user may observe false-negative results if sufficient slide specimen heating with Citra Plus Antigen Retrieval solution is not obtained.

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.

InSite™ Her-2/neu primary antibody incubation with tissue sections should be performed at 20°C - 26°C. Use of temperatures other 20°C - 26°C may result in false negative or false positive results.

Improper storage of control slides may result in negative or improper staining. Also, false negativity may arise due to several reasons including loss of epitope on the tissue specimen.

Use of this antibody on specimens fixed in fixatives other than neutral buffered formalin has not been validated.

**XV. Performance Characteristics:**

**A. Specificity**

**(1) Antibody Specificity**

Specificity of the InSite™ Her-2/neu Monoclonal Antibody was demonstrated by Western Blot analysis of two cell lines, SK-BR3 and AU565, both known to over-express the Her-2 receptor. In both cell lines, one protein band (~195 kDA), closely corresponding to the known mass of the Her-2 receptor protein, was immunoprecipitated in protein cell extracts immobilized on nitrocellulose membrane. No cross-reactivity was seen with the 160 kDA Her-3 receptor protein moderately expressed by cell line SK-BR3. Faint detection of proteins of less than 118 kDA most likely represents degradation products of Her-2/neu.

**(2) Normal Tissue Reactivity**

A total of 30 normal tissues as recommended in “FDA Guidance for Submission of Immunohistochemistry Applications to the FDA” were evaluated. All tissues were formalin fixed and paraffin embedded and stained with one lot of InSite™ Her-2/neu and ancillary DAB staining reagents according to the staining procedure found in this package insert. The run was performed on the BioGenex i6000™ Automated Staining System. The results of this study can be found in Table 3 below.

**Table 3. Summary of Reactivity of InSite™ Her-2/neu in Normal Human Tissues.**

| <b>Tissue Type Tested</b> | <b>Results<br/>(# Negative/Total # of<br/>Sections Tested)</b> | <b>Cytoplasmic Staining Pattern Seen<br/>(# Reactive/Total # of Sections<br/>Tested)</b> |
|---------------------------|--|--|
| Cerebrum                  | Negative (3/3)   | Neurons, cytoplasm (1/3)   |
| Cerebellum                | Negative (3/3)   |  |
| Adrenal                   | Negative (3/3)   | Cortex cells, cytoplasm (2/3)  |
| Ovary                     | Negative (3/3)   | Exocrine cells, cytoplasm (3/3)  |
| Pancreas                  | Negative (3/3)   |  |
| Parathyroid               | Negative (1/1)   |  |
| Pituitary                 | Negative (1/1)   |  |
| Testis                    | Negative (3/3)   | Parafollicular cells, cytoplasm (1/3)  |
| Thyroid                   | Negative (3/3)   |  |
| Breast                    | Negative (3/3)   |  |
| Spleen                    | Negative (3/3)   |  |
| Tonsil                    | Negative (3/3)   | Blastic Stem Cells, cytoplasm (3/3)  |
| Thymus                    | Negative (3/3)   |  |
| Bone Marrow               | Negative (3/3)   |  |
| Lung                      | Negative (3/3)   |  |

|                  |                |   |
|------------------|----------------|---|
| Heart            | Negative (3/3) |   |
| Esophagus        | Negative (3/3) | Gland epithelial cells, cytoplasm (2/3) |
| Stomach          | Negative (3/3) | Epithelial cells, cytoplasm (2/3)       |
| Small Intestine  | Negative (3/3) | Epithelial cells, cytoplasm (2/3)       |
| Colon            | Negative (3/3) | Epithelial cells, cytoplasm (2/3)       |
| Liver            | Negative (3/3) | Hepatocytes, cytoplasm (1/3)            |
| Salivary Gland   | Negative (3/3) | Ducts, cytoplasm (2/3)                  |
| Kidney           | Negative (3/3) | Tubules, cytoplasm (1/3)                |
| Prostate         | Negative (3/3) |   |
| Uterus           | Negative (3/3) |   |
| Cervix           | Negative (3/3) |   |
| Skeletal         | Negative (3/3) |   |
| Skin             | Negative (3/3) | Sebaceous glands, cytoplasm (2/3)       |
| Peripheral Nerve | Negative (3/3) |   |
| Mesothelial      | Negative (3/3) |   |

All of the above normal human tissues showed negative reactivity to BioGenex InSite™ Her-2/neu staining. As documented in the third column of Table 3, cytoplasmic reactivity with BioGenex InSite™ Her-2/neu was seen in 13 of the 30 tissue types tested.

#### **B. Reproducibility**

The first three of the following reproducibility studies were carried out using formalin-fixed, paraffin-embedded tissue sections selected from five different breast cancer specimens, having known Her-2/neu immunostaining scores from 0 through 3+. All tissues were stained with one lot of InSite™ Her-2/neu antibody and DAB detection reagents on a BioGenex i6000™ Automated Staining System, following the staining procedure described in this package insert. Each specimen was run in a masked, randomized fashion, with one slide tested with the negative reagent control to assess background staining.

##### **(1) Intra-Run Reproducibility**

Intra-run reproducibility of staining with InSite™ Her-2/neu was tested on triplicate slides of breast tumor tissue sections taken from each of five different formalin-fixed, paraffin-embedded tissue blocks. Uniform reproducibility in semi-quantitative immunostaining intensity scores was seen across all slides of each specimen evaluated in the same run.

## (2) Inter-Run Reproducibility

Inter-run reproducibility of staining with InSite™ Her-2/neu was carried out on three separate days, using breast tumor tissue sections taken from each of five different formalin-fixed, paraffin-embedded tissue blocks. The results of this study are presented in Table 4 below.

**Table 4. Summary of Staining Intensities of InSite™ Her-2/neu on Breast Tissue Sections Obtained in Three Separate Runs.**

|                       | First Run (Day 1) |      | Second Run (Day 2) |      | Third Run (Day 3) |      |
|-----------------------|-------------------|------|--------------------|------|-------------------|------|
|                       | Her-2/neu*        | NC** | Her-2/neu*         | NC** | Her-2/neu*        | NC** |
| Quality Control Sides | 3+                | 0    | 3+                 | 0    | 3+                | 0    |
| S98-388               | 0                 | 0    | 0                  | 0    | 0                 | 0    |
| S97-3352A             | 0 ~ 1+            | 0    | 0                  | 0    | 0 ~ 1+            | 0    |
| S97-229               | 1+ ~ 2+           | 0    | 1+ ~ 2+            | 0    | 1+                | 0    |
| S97-1324A             | 2+ ~ 3+           | 0    | 2+                 | 0    | 2+                | 0    |
| S97-2357B             | 3+                | 0    | 3+                 | 0    | 3+                | 0    |

\* BioGenex InSite™ Her-2/neu

\*\* NC, Negative Control

Mild variability of inter-run reproducibility was seen as inferred from reproducibility of semi-quantitative immunostaining intensity score. One specimen returned mildly discrepant semi-quantitative immunostaining intensity scores of 1+ ~ 2+, 1+ ~ 2 and 1+ across three runs. However, such variability, as seen between runs in this study, is well documented. Low reproducibility in semi-quantitative immunostaining score is typical within the scoring range of 1+ ~ 2+. Therefore, it is commonly accepted that semi-quantitative scores of 2+ be reevaluated by additional observers or FISH analysis before final diagnostic decision.<sup>29, 30, 31, 32</sup>

## (3) Manual vs. Automated Methodology Reproducibility

A single section from each of the five breast tumor formalin-fixed, paraffin-embedded tissue blocks was used and subjected to immunohistochemical staining using either a manual or automated protocol. The results of this study are presented in Table 5 below.

**Table 5. Summary of Staining Intensities of InSite™ Her-2/neu on Breast Tissue Sections Obtained in a Manual vs. Automated Assay System.**

|                       | Manual      |       | Automated*  |       |
|-----------------------|-------------|-------|-------------|-------|
|                       | Her-2/neu** | NC*** | Her-2/neu** | NC*** |
| Quality Control Sides | 3+          | 0     | 3+          | 0     |
| S98-388               | 0           | 0     | 0           | 0     |
| S97-3352A             | 0           | 0     | 0           | 0     |
| S97-229               | 1+          | 0     | 1+ ~ 2+     | 0     |
| S97-1324A             | 2+          | 0     | 2+          | 0     |
| S97-2357B             | 3+          | 0     | 3+          | 0     |

\* Performed on a BioGenex i6000™ Automated Staining System

\*\* BioGenex InSite™ Her-2/neu

\*\*\* NC, Negative Reagent Control

Mild variability between manual vs. automated methodology reproducibility was seen. One specimen returned mildly discrepant semi-quantitative immunostaining intensity scores of 1+ and 1+ ~ 2+ for the manual and automated protocol, respectively. However, such variability, as seen across protocol in this study, is well documented. Low reproducibility in semi-quantitative immunostaining score is typical within the scoring range of 1+ or 2+. Therefore, it is commonly accepted that semi-quantitative scores of 2+ be reevaluated by additional observers or FISH analysis before a final diagnostic decision is made.<sup>29, 30, 31, 32</sup>

**(4) Lot-to-Lot Reproducibility**

Three independent lots of InSite™ Her-2/neu kit were made according to manufacturing procedures. Cell line control slides with multiple cell lines with reactivities that ranged in semi-quantitative intensity scores from 0 to 3+ were used for testing. For each lot, three slides were used with one slide as negative control. The results of this study are presented in Table 6 below.

**Table 6. Summary of Staining Intensities of Three Lots of InSite™ Her-2/neu on a Cell Line Control Slide.**

|     | RD1340904 |            |            | RD13490B |            |            | RD13490C |            |            |
|-----|-----------|------------|------------|----------|------------|------------|----------|------------|------------|
|     | SK-BR3    | MDA-MB-175 | MDA-MB-231 | SK-BR3   | MDA-MB-175 | MDA-MB-231 | SK-BR3   | MDA-MB-175 | MDA-MB-231 |
| 1*  | 3+        | 1+ ~ 2+    | 0          | 3+       | 1+         | 0          | 3+       | 1+         | 0          |
| 2*  | 3+        | 1+ ~ 2+    | 0          | 3+       | 1+         | 0          | 3+       | 1+         | 0          |
| 3** | 0         | 0          | 0          | 0        | 0          | 0          | 0        | 0          | 0          |

\* Stained with BioGenex InSite™ Her-2/neu

\*\* Stained with Negative Reagent Control

Mild variability of lot-to-lot reproducibility was seen. One specimen returned mildly discrepant semi-quantitative immunostaining intensity scores of 1+ and 1+ ~ 2+ across three lots. However, such variability, as seen across lots in this study, is well documented. Low reproducibility in semi-quantitative immunostaining score is typical within the scoring range of 1+ or 2+. Therefore, it is commonly accepted that semi-quantitative scores of 2+ be reevaluated by additional observers or FISH analysis before a final diagnostic decision is made.<sup>29, 30, 31, 32</sup>

### (5) Inter-Laboratory Reproducibility

The reproducibility of InSite™ Her-2/neu performance between laboratories was tested. For this particular study, all investigators followed a common manual Immunohistochemistry staining procedure, as described in this package insert. The laboratories were also provided with the same lot of InSite™ Her-2/neu and ancillary DAB staining reagents. In brief, three different, geographically distinct laboratories were each provided with unstained breast tumor tissue sections from the same 30 different cases of formalin-fixed, paraffin-embedded tissue blocks. These cases were comprised of 10 each of 3+ and 2+ Her-2/neu staining scores, and 5 each of 1+ and 0 Her-2/neu staining scores. The results of this study are presented in Table 7 below.

**Table 7.  
Summary of  
Inter-  
Laboratory  
Concordance  
for InSite™  
Her-2/neu.**

|         | LABS |   |       | Counts |
|---------|------|---|-------|--------|
|         | A    | B | C     |        |
| RESULTS | -    | - | -     | 5      |
|         | -    | - | +     | 1      |
|         | -    | + | -     | 2      |
|         | -    | + | +     | 1      |
|         | +    | + | -     | 2      |
|         | +    | + | +     | 19     |
|         |      |   | TOTAL | 30     |

Most of the discrepant results were between 1+ and 2+ staining scores. In general however, very good reproducibility of positive vs. negative scores across the three laboratories was observed for all breast tumor tissue specimens used. This is based on the concordance of staining results between labs which ranged from 83% - 90%, with Labs A and B at 90%; Labs A and C at 86%; and Labs B and C at 83%.

### C. Agreement with DakoCytomation HercepTest®

The objective of this study was to determine the suitability of BioGenex InSite™ Her-2/neu as an alternative to DakoCytomation HercepTest® for use as an aid in the determination of appropriateness of Herceptin® therapy in breast cancer patients. Formalin-fixed, paraffin-embedded breast tumor specimens were used. One set of specimens was stained with HercepTest®, whilst the second matching set was stained with InSite™ Her-2/neu. Two lots of HercepTest® kit and two lots of InSite™ Her-2/neu antibody and reagents were used for this study. Staining and scoring was carried out by two histotechnologists and two pathologists, respectively. No statistically significant difference could be attributed to the results obtained for these different groups, thus the data from them were pooled. Staining with HercepTest® was carried out according to the manufacturer's package insert, following a manual IHC procedure. Conversely, staining with InSite™ Her-2/neu was performed on a BioGenex i6000™ Automated Staining System, based on the procedure in this package insert. This study was performed in a single blinded manner. Both a 2x2 and 3x3 concordance analysis was conducted. The acceptance criteria was defined as >75% overall concordance between the two tests within a 95% confidence interval.

A total of 352 identical slide pairs of unique breast tumor specimen tissue were evaluated in this study. Positive and negative scoring specimens were equally represented. Agreement was determined by staining one slide each of an identical breast tumor specimen pair with either BioGenex InSite™ Her-2/neu Monoclonal Antibody and DAB detection reagents or HercepTest®.

Scores were interpreted as negative if the staining intensity score was 0 or 1+. Scores interpreted as 2+ or 3+ were reported as positive for Her-2/neu receptor expression.

#### (I) 2x2 Concordance

Table 8 below summarizes the immunostaining results obtained from 352 pairs of identical breast tumor specimens when stained

with both InSite™ Her-2/neu and HercepTest® and scored as either 'positive' (2+ or 3+) or 'negative' (0 or 1+).

**Table 8: 2x2 Concordance Results.**

| InSite™ Her-2/neu | HercepTest® |     | Total |
|-------------------|-------------|-----|-------|
|                   | -           | +   |       |
| -                 | 128         | 7   | 135   |
| +                 | 36          | 181 | 217   |
| Total             | 164         | 188 | 352   |

Concordance =87.8% (83.9%, 91.0%); 95% Confidence Interval

The overall 2x2 concordance of the InSite™ Her-2/neu with HercepTest® is 87.8% (309/352), with a two-side 95% confidence interval of 83.9% to 91.0%. The null hypothesis,  $H_0$ : concordance  $\leq$  75%, was rejected with p-value  $<$  0.00001.

Percent Positive Agreement (the percentage of specimens scored as positive by HercepTest® that were also scored positive by InSite™ Her-2/neu) was 96.3% (181/188), with a 95% confidence interval of 92.5% to 98.5%. Percent Negative Agreement (the percentage of specimens scored negative by HercepTest® that were also scored negative by InSite™ Her-2/neu) was 78.0% (128/164), with a 95% confidence interval of 70.9% to 84.1%.

The Kappa measure of agreement was 0.752, with a 95% confidence interval of 0.683 to 0.820. The sample value of Kappa measure has a large-sample normal distribution. Its estimated asymptotic standard error is 0.0350. The null hypothesis,  $H_0$ : agreement is no better than chance, was rejected with p-value  $<$  0.00001.

**(2) 3x3 Concordance**

Table 9 below summarizes the immunostaining results obtained from 352 pairs of identical breast tumor specimens when stained with both InSite™ Her-2/neu and HercepTest® and scored as either 3+, 2+ or 'negative' (0 or 1+).

**Table 9: 3x3 Concordance Results.**

| InSite™ Her-2/neu | HercepTest™ |    |    | Total |
|-------------------|-------------|----|----|-------|
|                   | -           | 2+ | 3+ |       |
| -                 | 128         | 5  | 2  | 135   |
| 2+                | 25          | 80 | 9  | 114   |
| 3+                | 11          | 14 | 78 | 103   |

|              |     |    |    |     |
|--------------|-----|----|----|-----|
| <b>Total</b> | 164 | 99 | 89 | 352 |
|--------------|-----|----|----|-----|

Concordance = 81.3% (76.8%, 85.2%); 95% Confidence Interval

The overall 3x3 concordance of the InSite™ Her-2/neu with HercepTest® is 81.3% (286/352), with a two-side 95% confidence interval of 76.8% to 85.2%. The null hypothesis, H<sub>0</sub>: concordance ≤ 75%, was rejected with p-value = 0.0022.

Percent Positive Agreement (the percentage of specimens scored as 2+ by HercepTest® that were also scored 2+ by InSite™ Her-2/neu) for scores of 2+ was 80.8% (80/99), with a 95% confidence interval of 71.7% to 88.0%. The Percent Positive Agreement of InSite™ Her-2/neu for scores of 3+ was 87.6% (78/89), with a 95% confidence interval of 79.0% to 93.7%. Percent Negative Agreement (the percentage of specimens scored negative by HercepTest® that were also scored negative by InSite™ Her-2/neu) was 78.0% (128/164), with a 95% confidence interval of 70.9% to 84.1%.

The Kappa measure of agreement was 0.714, with a 95% confidence interval of 0.653 to 0.776. The sample value of Kappa measure has a large-sample normal distribution. Its estimated asymptotic standard error is 0.0313. The null hypothesis, H<sub>0</sub>: agreement is no better than chance, was rejected with p-value < 0.00001.

In conclusion, as can be seen in Table 9, there are numerous discrepant results. Many of these lie in the 2+ range defined as “weak to moderate complete membrane staining observed in more than 10% of tumor cells”. Previous work by independent investigators observed that specimens with a Her-2/neu score of 2+ achieved a low percentage of inter-observer agreement.<sup>20, 29, 30, 31</sup> Other factors, such as mild run to run variability in the immunostaining process over a period of time, may contribute to the difficulty of defining the 2+ staining intensity range. Therefore, it is generally accepted that specimens giving an ambiguous Her-2/neu score of 2+ be reevaluated by multiple observers and/or subjected to FISH analysis.<sup>29, 30, 31, 32</sup>

A false negative immunostaining score result (0 or 1+) would result in patients who may not be selected for therapy with Herceptin® or Herceptin® in combination with other therapies. Conversely, a false positive immunostaining score (2+ or 3+) would result in patients receiving Herceptin® therapy despite its unknown benefit(s).

Adverse side effects associated with Herceptin<sup>®</sup> therapy have been reported, which include infusional toxicity and cardiotoxicity.<sup>5,6</sup>

## **XVI. Troubleshooting:**

### **A. Overstaining**

1. Incubation time of primary antibody too long.
2. Incubation temperature of primary antibody too high.
3. Substrate incubation too long.
4. Slides inadequately rinsed.

### **B. Weak staining on all slides**

1. Omission of recommended pretreatment: i.e., Antigen Retrieval pretreatment or inappropriate use of pretreatment method.
2. Incubation time with primary antibody too short.
3. Incubation temperature with primary antibody too low.
4. Substrate too old.
5. Too much rinse buffer left on slides causing excessive dilution of reagents.
6. Incompatible counterstain or mounting media which dissolves reaction product.
7. Incorrect deparaffinization of tissue.

### **C. No staining on any slide**

1. Omission of primary antibody.
2. Incorrect procedure: reagents used in the wrong order.
3. Incorrect preparation of substrate-chromogen solution.
4. Sodium azide contamination in rinse solution.

### **D. Staining positive control slide only (test slide shows no signal)**

1. No antigen present or level too low for detection by staining procedure.
2. Improper preparation of specimen tissue causing denaturation of antigen.
3. Immunoreactivity diminished or destroyed during tissue processing due to high temperature. For initial validation of Immunohistochemistry, a set of controls should be run with the antibody and tissue to be tested. Negative controls should show no staining if the reaction is specific to the antigen.

### **E. Background**

1. Endogenous biotin in tissue requires pretreatment with an avidin-biotin block.
2. Nonspecific reagent binding in tissue. Necrosis in tissues or improper fixation. May need an additional blocking step.
3. Inadequate rinsing. (Use fresh and adequate washing buffers.)
4. Incomplete deparaffinization.

5. Substrate incubation too long.
6. Tissue dried out during staining protocol.
7. Antigen diffusion prior to fixation. Avoid delays in processing of tissue.
8. For formalin-fixed tissues, factors such as time, temperature and pH of fixation can cause antigens to be masked by aldehyde cross-linking and an increased hydrophobicity of tissue. This can lead to non-specific binding. It may be possible to recover antigenicity with Antigen Retrieval pretreatment or to reduce background with a blocking reagent.

**F. If tissue sections wash off slide, make sure slides are positively charged (i.e., silanized or coated with polylysine or equivalent material).**

Remove additives from water bath during transfer of tissue sections to slides. If you have questions regarding either the use of the reagents in this kit or the results obtained, contact BioGenex Technical Support at Tel. No. (800) 421-4149.

**XVII. Bibliography:**

- 1) Akiyama, T. et. al. The product of the human c-erbB-2 gene: a 185kDA glycoprotein with tyrosine kinase activity. *Science* 232(4758): 1644-6, 1986.
- 2) Bast, R.C. et. al. 2000 update of recommendations for the use of tumor markers in breast and colorectal cancer: clinical practice guidelines of the American Society of Clinical Oncology. *J Clin. Oncol.* 19: 1865-1878, 2001
- 3) Brandt, B.H. et. al. c-erbB-2/EGFR as dominant heterodimerization partners determine a mutagenic phenotype in human breast cancer cells. *FASEB.* 13(14): 1939-1949, 1990.
- 4) Brown, R.E. HER-2/neu-positive breast carcinoma: molecular concomitants by proteomic analysis and their therapeutic implications. *Annual Clin. Lab. Science* 32(1):12-21, 2002.
- 5) Burstein, H.J., Harris, L.N., Gelman, R., Lester, S.C., Nunes, R.A., Kaelin, C.M., Parker, L.M., Ellisen, L.W., Kuter, I., Gadd, M.A., Christian, R.L., Kennedy, P.R., Borges, V.F., Bunnell, C.A., Younger, J., Smith, B.L., and Winer, E.P. Preoperative therapy with trastuzumab and paclitaxel followed by sequential adjuvant doxorubicin/cyclophosphamide for HER2 overexpressing stage II and III breast cancer: a pilot study. *Journal of Clinical Oncology* 21(1): 46-53, 2003.
- 6) Burstein, H.J., Harris, L.N., Marcom, P.K., Lambert-Falls, R., Havlin, K., Overmoyer, B., Friedlander Jr., R.J., Gargiulo, J., Strenger, R., Vogel, C.L., Rya, P.D., Ellis, M.J., Nunes, R.A., Bunnell, C.A., Campos, S.M., Hallor, M., Gelman, R. and Winer, E.P. Trastuzumab and vinorelbine as first-line therapy for HER2-overexpressing metastatic breast cancer: multicenter phase II trial with clinical outcomes, analysis of serum tumor markers as

- predictive factors, and cardiac surveillance algorithm. *Journal of Clinical Oncology* 21(15): 2889-2895, 2003.
- 7) Center for Disease Control. Decontamination of Laboratory Sink Drains to Remove Azide Salts. Center for Disease Control Manual Guide--Safety Management, No. CDC-22, Atlanta, Georgia. April 30, 1976.
  - 8) Cobleigh, M.A., et. al. Multinational study of the efficacy and safety of humanized anti-Her-2 monoclonal antibody in women who have Her-2 overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin. Oncol.* 9: 2639-48, 1999.
  - 9) College of American Pathologists (CAP) Certification Program for Immunohistochemistry. Northfield, IL. <http://www.cap.org> (800) 323-4040.
  - 10) Corbett, I.P. et. al. NCL-CB11, a new monoclonal antibody recognizing the internal domain of the c-erbB-2 oncogene protein effective for use on formalin-fixed, paraffin-embedded tissue. *J Pathology* 161: 15-25, 1990.
  - 11) DAKO A/S. FDA PMA# P980018. Summary of Safety and Effectiveness Data. 1998. [online] Available: <http://www.fda.gov/cdrh/pdf/p980018.pdf> [2004].
  - 12) Dickson, R.B. and Lippman, M.E. Genes, Oncogenes and Hormones. Boston, Kluwer Academic Publishers, 1992.
  - 13) Earp, H.S. et. al. Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research. *Breast Cancer Res. Treat.* 35(1): 115-32, 1995.
  - 14) Heintz, N.H., et al. Amplification of the c-erbB-2 oncogene and prognosis of breast adenocarcinoma. *Arch. Pathol. Lab. Med.* 114: 160-163, 1990.
  - 15) Keatings, L. et al. C-erbB-2 oncoprotein expression in mammary and extramammary Paget's disease: an immunohistochemical study. *Histopathology.* 17: 234-247, 1990.
  - 16) Kiernan JA. Histological and Histochemical Methods: Theory and Practice. New York: Pergamon Press 1981.
  - 17) Latta, E.K. et. al. The Role of Her-2/neu overexpression/amplification in the progression of ductal carcinoma in situ to invasive carcinoma of the breast. *Mod. Pathology* 15(12): 1318-1325, 2002.
  - 18) Nadji M. and Morales, A.R. Immunoperoxidase, part 1: the techniques and its pitfalls. *Lab. Med.* 14: 767-770, 1983.
  - 19) National Institute for Occupational Safety and Health, (NIOSH), Rockville, MD. Explosive azide hazard, Publication No. 78-127, 1976.
  - 20) Nichols, DW., Wolff, DJ., Self, D., Metcalf, JS., Jabobs, D., Kneuper-Hall, R., and Cate 4<sup>th</sup>, JC. A testing algorithm for determination of HER2 status in patients with breast cancer. *Annual Clinical Lab. Science* 32(1): 3-11, 2002.
  - 21) O'Leary T.J., et. al. Quality assurance for immunocytochemistry; proposed guideline. MM4-P. National Committee for Clinical Laboratory Standards (NCCLS). Wayne, PA. 1997; 1-46.
  - 22) Omata, M., et. al. Nonimmunologic binding of horseradish peroxidase to hepatitis B surface antigen. A possible source of error in immunohistochemistry. *Amer. J Clin. Pathol.* 73:626-632, 1980.

- 23) Rodriguez, G.C., et. al. The effect of antibodies and immunotoxins reactive with HER-2/neu on the growth of ovarian and breast cancer cell lines. *Amer. J Obstet. Gynecol.* 168(1.1): 238-232, 1993.
- 24) Riese 2<sup>nd</sup>, D.J. and Stern, D.F. Specificity within the EGF family/ErbB receptor family signaling network. *Bioessays* 20(1): 41-8, 1998.
- 25) Sheehan, D.C. and Hrapchak, B.B. Theory and Practice of Histotechnology. St. Louis: C.V. Mosby Co. 1980.
- 26) Shih, C., et. al. Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature* 290(5803): 261-4, 1981.
- 27) Siegall, C.B., et. al. HER4 expression correlates with cytotoxicity directed by a heregulin-toxin fusion protein. *J Biol. Chem.* 270(13): 7625-30, 1995.
- 28) Slamon, D.J., and Clark, G.M. Amplification of c-erbB-2 and aggressive human breast tissues? *Science* 240:1795-1798, 1988.
- 29) Thomson, T.A., et. al. HER-2/neu in breast cancer: interobserver variability and performance of immunohistochemistry with 4 antibodies compared with fluorescent in situ hybridization. *Mod. Pathology* 14 (11): 1079-1086, 2001.
- 30) Tsuda, H., et. al. Detection of Her-2/neu (c-erbB-2) DNA amplification in primary breast carcinoma, Interobserver reproducibility and correlation with immunohistochemical Her-2 overexpression. *Cancer* 92(12): 2965-74, 2001.
- 31) Tsuda, H., et. al. Concordance in judgments among c-erbB-2 (HER-2/neu) overexpression detected by two immunohistochemical tests and gene amplification detected by Southern Blot hybridization in breast carcinoma. *Pathol. Int.* 51(1): 26-32, 2001.
- 32) Tsuda, H., et. al. Evaluation of interobserver agreement in scoring immunohistochemical results of Her-2/neu (c-erbB-2) expression detected by HercepTest, Nichirei polyclonal antibody, CB11 and TAB 250 in breast carcinoma. *Pathol. Int.* 52(2): 126-34, 2002.
- 33) Tzahar, E., et al. A hierarchical network of inter-receptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol. Cell Biol.* 10: 5276-87, 1996.
- 34) U.S. Congress. Clinical Laboratory Improvement Amendments of 1988: Final Rule, 57 FR 7163, February 28, 1992.
- 35) Wright, C., et. al. Expression of c-erbB-2 oncoprotein: a prognostic indicator in human breast cancer. *Cancer Res.* 49: 2087-2090, 1989.
- 36) Yee, L.D., et al. Oncogene structure, function, and expression in breast cancer. *Sem. Diag. Pathol.* 6: 110-125, 1989.

OptiMax® and MultiLink® are registered trademarks of BioGenex.  
 EZ-DeWax™ and i6000™ are trademarks of BioGenex.

Citra Plus Antigen Retrieval is covered by U.S. Patent No. 5,578,452; Japanese Patent No. 3108099; and European Patent No. 0607422.

Microwave Antigen Retrieval is covered by U.S. Patent No. 5,244,787.

EZ-DeWax™ is covered by U.S. Patent No. 6,632,598; European Patent No. 0698118; and Equivalent Foreign Patents Pending.

HerceptTest® and Herceptin® are trademarks of Genentech, Inc., subject to licenses held by DakoCytomation Denmark A/S and F. Hoffmann-La Roche, Ltd.

### Glossary of Graphical Symbols Used in the Labels

|   |                                   |   |                                   |   |  |   |                               |
|---|-----------------------------------|---|-----------------------------------|---|--|---|-------------------------------|
|  | Corrosive<br>(French)<br>(German) |  | Flammable<br>(French)<br>(German) |  | Harmful/Irritant<br>(French)<br>(German) |  | Toxic<br>(French)<br>(German) |
|---|-----------------------------------|---|-----------------------------------|---|--|---|-------------------------------|

|   |   |   |  |   |   |
|---|---|---|--|---|---|
|    | Authorized Representative in the European Community<br>(French)<br>(German) |    | Consult Instructions for Use<br>(French)<br>(German)       |    | Manufacturer<br>(French)<br>(German)            |
|    | Batch Code<br>(French)<br>(German)  |    | Contains Sufficient for <N> Tests<br>(French)<br>(German)  |    | Temperature Limitations<br>(French)<br>(German) |
|  | Catalog / Code Number<br>(French)<br>(German)                               |  | In Vitro Diagnostic Medical Device<br>(French)<br>(German) |  | Use By<br>(French)<br>(German)                  |



BioGenex Laboratories, Inc.  
4600 Norris Canyon Road  
San Ramon, CA 94583

Tel. No. (925) 275-0550  
FAX No. (925) 275-0580  
Toll Free (800) 421-4149 (U.S. Only)  
Web Site: [www.biogenex.com](http://www.biogenex.com)



Emergo Europe  
Molenstraat 15  
2513 BH The Hague  
The Netherlands  
Tel. No. +31 (70) 345-8570  
FAX No. +31 (70) 346-7299

©Copyright 2004. BioGenex Laboratories, Inc.