DakoCytomation

CE

# *HER2* FISH pharmDx™ Kit

Code No. K 5331

**4th edition**

Direct fluorescence in situ hybridization assay for quantitative determination of *HER2* gene amplification in formalin-fixed, paraffin-embedded breast cancer tissue. The assay is indicated as an aid in the assessment of patients for whom Herceptin™ treatment is being considered. Results from the *HER2* FISH pharmDx™ Kit are intended for use as an adjunct to the clinicopathologic information currently used for estimating prognosis in stage II, node-positive breast cancer patients.

The kit contains reagents sufficient for 20 tests.

## Intended Use

*HER2* FISH pharmDx™ Kit is a direct fluorescence in situ hybridization (FISH) assay designed to quantitatively determine *HER2* gene amplification in formalin-fixed, paraffin-embedded breast cancer tissue specimens.

*HER2* FISH pharmDx™ Kit is indicated as an aid in the assessment of patients for whom Herceptin™ (trastuzumab) treatment is being considered.

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

## Summary and Explanation

The human *HER2* gene (also known as *ERBB2* or *NEU*) is located on chromosome 17 and encodes the 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, 2). The *HER2* gene is present in 2 copies in all normal diploid cells.

In a fraction of patients with breast cancer, the *HER2* gene is amplified as part of the process of malignant transformation and tumour progression (3-8). *HER2* gene amplification generally leads to over expression of the HER2 protein on the surface of breast cancer cells (9).

Amplification of the *HER2* gene and/or over expression of its protein have been demonstrated in 25-30% of breast cancers. This up-regulation is associated with poor prognosis, increased risk of recurrence, and shortened survival. Several studies have shown that HER2 status correlates with sensitivity or resistance to certain chemotherapy regimens (10).

Demonstration of high HER2 protein over expression or *HER2* gene amplification is essential for initiating therapy with Herceptin™, a monoclonal antibody to HER2 protein. Clinical studies have shown that patients whose tumours have high HER2 protein over expression and/or amplification of the *HER2* gene benefit most from Herceptin™ (11).

## Principle of Procedure

*HER2* FISH pharmDx™ Kit contains all reagents required to complete a FISH procedure for formalin-fixed, paraffin-embedded tissue section specimens.

*For interactive e-learning please use the HER2* FISH pharmDx™ *e-learning program designed to supply laboratory technicians, pathologists and scientists globally with an accurate and fast knowledge of how to achieve optimal results using HER2* FISH pharmDx™*: www.dakocytomation.com/learning*

After deparaffinization and rehydration, specimens are heated in Pre-Treatment Solution for 10 minutes. The next step involves a proteolytic digestion using ready-to-use Pepsin at room temperature for 5-15 minutes. Following the heating and proteolytic pre-treatment steps, this kit employs a ready-to-use FISH Probe Mix based on a combination of PNA (peptide nucleic acid) (12) and DNA technology. This Probe Mix consists of a mixture of Texas Red-labelled DNA probes covering a 218 kb region including the *HER2* gene on chromosome 17, and a mixture of fluorescein-labelled PNA probes targeted at the centromeric region of chromosome 17 (CEN-17). The specific hybridization to the two targets results in formation of a distinct red fluorescent signal at each *HER2* gene locus and a distinct green fluorescent signal at each chromosome 17 centromere. To diminish background staining, the Probe Mix also contains unlabelled PNA blocking probes. After a stringent wash, the specimens are mounted with Fluorescence Mounting Medium containing DAPI and coverslipped. Using a fluorescence microscope equipped with appropriate filters (see Appendix 3), tumour cells are located, and enumeration of the red *(HER2)* and green (CEN-17) signals is conducted. Then the *HER2*/CEN-17 ratio is calculated. Normal cells in the analyzed tissue section will serve as an internal positive control of pre-treatment and hybridization efficiency. For details see the Interpretation of Staining section.

## Reagents

**Materials provided**

The materials listed below are sufficient for 20 tests (a test is defined as one 22 mm x 22 mm target area). The number of tests is based on the use of 5-8 drops (250 µL per slide of Vial 2, 10 µL per slide of Vial 3, and 15 µL per slide of Vial 5. The kit provides materials sufficient for 10 individual staining runs.

*HER2* FISH pharmDx™ Kit is shipped on dry ice. **To ensure that kit components have not been exposed to high temperatures during transport, dry ice should still be present upon receipt**. Note that some kit components may remain unfrozen, this will not affect the performance of the *HER2* FISH pharmDx™ Kit.

| | |
|---|---|
| **Vial 1** | PRE-TREATMENT SOLUTION (X 20)<br>**Pre-Treatment Solution (x 20)**<br>75 mL, concentrated x 20<br>MES (2-[*N*-morpholino]ethanesulphonic acid) buffer. |
| **Vial 2** | PEPSIN<br>**Pepsin**<br>5 mL, ready-to-use<br>Pepsin solution, pH 2.0; contains stabilizer and an antimicrobial agent. |
| **Vial 3** | *HER2*/CEN-17 PROBE MIX<br>***HER2*/CEN-17 Probe Mix**<br>0.2 mL, ready-to-use<br>Mix of Texas Red-labelled *HER2* DNA probes and fluorescein-labelled CEN-17 PNA probes; supplied in hybridization buffer with 45% formamide, stabilizer, and unlabelled PNA blocking probes. |
| **Vial 4** | STRINGENT WASH BUFFER (X 20)<br>**Stringent Wash Buffer (x 20)**<br>150 mL, concentrated x 20<br>SSC (saline-sodium citrate) buffer with detergent. |
| **Vial 5** | FLUORESCENCE MOUNTING MEDIUM<br>**Fluorescence Mounting Medium**<br>0.3 mL, ready-to-use<br>Fluorescence mounting medium with 100 µg/L DAPI (4',6-diamidine-2-phenylindole). |
| **Vial 6** | WASH BUFFER (X 20)<br>**Wash Buffer (x 20)**<br>500 mL, concentrated x 20<br>Tris/HCl buffer. |
| | COVERSLIP SEALANT<br>**Coverslip Sealant**<br>1 tube, ready-to-use<br>Solution for removable sealing of coverslips. |

**NOTE:** All reagents, including Pre-Treatment Solution, Stringent Wash Buffer, and Fluorescence Mounting Medium, are formulated specifically for use with this kit.

**Materials required but not provided**

**Laboratory reagents**

Distilled or deionized water

Ethanol, 96%

Xylene or xylene substitutes

**Laboratory equipment**

Absorbent wipes

Adjustable pipettes

Calibrated partial immersion thermometer (range 37-100 °C)

Calibrated surface thermometer (range 37-100 °C)

Coverslips (22 mm x 22 mm)

Forceps

Fume hood

Heating block or hybridization oven for denaturation (82 (±2) °C)

Humid hybridization chamber

Hybridization oven or incubator for overnight hybridization (45 (±2) °C)

Slides, DakoCytomation Silanized Slides, code No. S 3003, or poly-L-lysine-coated slides (see Specimen Preparation)

Staining jars or baths

Timer (capable of 2-15 minute intervals)

Water bath with lid (capable of maintaining 65 (±2) °C to 99 °C)

**Microscope equipment and accessories**

Filters for fluorescence microscope: DAPI and FITC/Texas Red double filter, or FITC and Texas Red mono filters - see Appendix 3 for details

Fluorescence microscope with a 100 watt mercury lamp is recommended

Microscope slide folder (cardboard tray for 20 slides with hinged cover or similar)

## Precautions

1. For in vitro diagnostic use.
2. For professional users.
3. Vial 1, Pre-Treatment Solution (x 20), contains 1-<20% 2-morpholinoethanesulphonic acid; Vial 2, Pepsin, contains 5-10% propan-2-ol; Vial 4, Stringent Wash Buffer (x 20), contains 1-<5% octoxinol; and Vial 6, Wash Buffer (x 20), contains 1-<20% trometamol. At product concentrations these substances do not require hazard labelling. Material Safety Data Sheets (MSDSs) are available for professional users on request.
4. Vial 2, Pepsin, contains pepsin A that may cause an allergic reaction.
5. Vial 3, *HER2*/CEN-17 Probe Mix contains 45% formamide and is labelled:
   Toxic.
   R61 May cause harm to the unborn child.
   S45 In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).
   S53 Avoid exposure – obtain special instructions before use.
   S60 This material and/or its container must be disposed of as hazardous waste.
   As a main rule, persons under 18 years of age are not allowed to work with this product. Users must be carefully instructed in the proper working procedure, the dangerous properties of the product and the necessary safety instructions. Please refer to the Material Safety Data Sheet (MSDS) for additional information.

6. Coverslip Sealant contains 60-100% naphtha (petroleum), hydrotreated light, and is labelled:
   Extremely flammable.
   Dangerous for the environment.
   R11 Highly flammable.
   R51/53 Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.
   S9 Keep container in a well-ventilated place.
   S16 Keep away from sources of ignition – No smoking.
   S35 This material and its container must be disposed of in a safe way.
   S57 Use appropriate container to avoid environmental contamination.
   S61 Avoid release to the environment. Refer to special instructions/safety data sheets.
   Please refer to the Material Safety Data Sheet (MSDS) for additional information.
7. Specimens, before and after fixation, and all materials exposed to them, should be handled as if capable of transmitting infection and should be disposed of with proper precautions (16). Never pipette reagents by mouth and avoid contacting the skin and mucous membranes with reagents and specimens. If reagents come in contact with sensitive areas, wash with copious amounts of water.
8. Minimize microbial contamination of reagents to avoid erroneous results.
9. Incubation times and temperatures, or methods other than those specified, may give erroneous results.
10. Tissue fixation methods and thickness of specimen other than those specified may affect tissue morphology and/or signal intensity.
11. Avoid evaporation of *HER2*/CEN-17 Probe Mix during hybridization by ensuring sufficient humidity in the hybridization chamber.
12. Reagents have been optimally diluted. Further dilution may result in loss of performance.
13. Wear appropriate personal protective equipment to avoid contact with eyes and skin. Please refer to the Material Safety Data Sheet (MSDS) for additional information.

## Storage

Store in the dark at 2-8 °C. All reagents tolerate frozen storage. Freezing and thawing the reagents for each analysis does not affect performance.

The Pepsin, *HER2*/CEN-17 Probe Mix, and Fluorescence Mounting Medium (Vials 2, 3 and 5) may be affected adversely if exposed to heat. Do not leave these components at room temperature. The *HER2*/CEN-17 Probe Mix and Fluorescence Mounting Medium (Vials 3 and 5) may be affected adversely if exposed to excessive light levels. Do not store these components or perform analysis in strong light, such as direct sunlight.

Do not use the kit after the expiration date stamped on the kit box. If reagents are stored under conditions other than those specified in this package insert, the user must validate reagent performance (13).

There are no obvious signs to indicate instability of this product. Therefore, it is important to evaluate normal cells in the analyzed tissue section. If an unexpected fluorescence pattern is observed which cannot be explained by variations in laboratory procedures, and a problem with the *HER2* FISH pharmDx™ Kit is suspected, contact our Technical Services.

## Specimen Preparation

Specimens from biopsies, excisions or resections must be handled to preserve the tissue for FISH analysis. Standard methods of tissue processing for immunocytochemical staining should be used for all specimens (14).

### Paraffin-embedded sections

Only tissue preserved in neutral buffered formalin and paraffin-embedded are suitable for use. Specimens should e.g. 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 graded series of ethanol and xylene, followed by infiltration by melted paraffin held at no more than 60 °C. Properly fixed and embedded tissues will keep indefinitely prior to sectioning and slide mounting if stored in a cool place (15-25 °C) (14, 15). Other fixatives are not suitable.

Tissue specimens should be cut into sections of 4-6 µm.

The slides required for *HER2* gene amplification analysis and verification of tumour presence should be prepared at the same time. A minimum of 2 serial sections is recommended, 1 section for tumour presence stained with hematoxylin and eosin (H&E stain), and 1 section for *HER2* gene amplification analysis. It is recommended that tissue sections are mounted on DakoCytomation Silanized Slides, code No. S 3003, or poly-L-lysine-coated slides. Specimens should be analyzed within 4-6 months of sectioning when stored at room temperature (20-25 °C).

## INSTRUCTIONS FOR USE

*For interactive e-learning please use the HER2 FISH pharmDx™ e-learning program designed to supply laboratory technicians, pathologists and scientists globally with an accurate and fast knowledge of how to achieve optimal results using HER2 FISH pharmDx™: www.dakocytomation.com/learning*

## A. Reagent Preparation

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

### A.1 Pre-Treatment Solution

Crystals may occur in Vial 1, but they will dissolve at room temperature. Ensure that no crystals are present before preparation of reagent.

Dilute a sufficient quantity of Vial 1 (Pre-Treatment Solution x 20) by diluting the concentrate 1:20 in distilled or deionized water. Unused diluted solution may be stored at 2-8 °C for one month. Discard diluted solution if cloudy in appearance.

### A.2 Stringent Wash Buffer

Dilute a sufficient quantity of Vial 4 (Stringent Wash Buffer x 20) by diluting the concentrate 1:20 in distilled or deionized water. Unused diluted buffer may be stored at 2-8 °C for one month. Discard diluted buffer if cloudy in appearance.

### A.3 Wash Buffer

Dilute a sufficient quantity of Vial 6 (Wash Buffer x 20) by diluting the concentrate 1:20 in distilled or deionized water. Unused diluted buffer may be stored at 2-8 °C for one month. Discard diluted buffer if cloudy in appearance.

### A.4 Ethanol series

From a 96% ethanol solution, prepare 3 jars with 70%, 85%, and 96% ethanol, respectively. Store covered jars at room temperature or at 2-8 °C, and use for a maximum of 200 slides. Discard solutions if cloudy in appearance.

## 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).

If kit components are stored frozen, it is recommended to move the reagents to 2-8 °C the day before performing the analysis to allow proper temperature equilibration. All reagents should be equilibrated to the relevant temperature prior to use as follows:

**Vial 1:** The Diluted Pre-Treatment Solution should be equilibrated to **95-99 °C.**

**Vial 2:** Pepsin should be applied **at 2-8 °C** and kept cold continuously.

**Vial 3:** *HER2*/CEN-17 Probe Mix may be applied at any temperature from **2-25 °C.**

**Vial 4:** The Diluted Stringent Wash Buffer should be equilibrated to **65 (±2) °C** prior to use.

**Vial 5:** Fluorescence Mounting Medium may be applied at any temperature from **2-25 °C.**

**Vial 6:** The Diluted Wash Buffer should be equilibrated to room temperate **20-25 °C.**

**The Coverslip Sealant** may be applied at any temperature from **2-25 °C.**

**All steps must be performed at the outlined temperature.**

The procedure includes a number of dehydrations followed by drying of the tissue sections. Ensure that tissue sections are completely dry before proceeding to the next step. Do not allow tissue sections to dry during the other procedural steps.

If the staining procedure has to be interrupted, slides may be kept in Wash Buffer after the deparaffinization step for up to 1 hour at room temperature (20-25 °C) without affecting the results.

### B.2 Treatment of tissues prior to staining

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

1. Place slides in a xylene bath and incubate for 5 (±1) minutes. Change baths and repeat once.
2. Tap off excess liquid and place slides in 96% ethanol for 2 (±1) minutes. Change baths and repeat once.
3. Tap off excess liquid and place slides in 70% ethanol for 2 (±1) minutes. Change baths and repeat once.
4. Tap off excess liquid and place slides in diluted Wash Buffer (see INSTRUCTIONS FOR USE, Section A.3) for a minimum of 2 minutes. Commence staining procedure as outlined in Section B.3, Step 1, Pre-Treatment.

Xylene and alcohol solutions should be changed after 200 slides or less.

Xylene substitutes may be used.

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

### B.3 Staining protocol

**DAY 1**

**Step 1: Pre-Treatment**

Fill staining jars, e.g. Coplin jars, with the diluted Pre-Treatment Solution (see INSTRUCTIONS FOR USE, Section A.1). Place staining jars containing Pre-Treatment Solution in water bath. Heat water bath and the Pre-Treatment Solution to 95-99 °C. Measure temperature inside jar with a calibrated thermometer to ensure correct temperature. Cover jars with lids in order to stabilize the temperature and avoid evaporation.

Immerse the room temperature deparaffinized sections into the preheated Pre-Treatment Solution in the staining jars. Re-check temperature and incubate for 10 (±1) minutes at 95-99 °C.

Remove the entire jar with slides from the water bath. Remove lid and allow the slides to cool in the Pre-Treatment Solution for 15 minutes at room temperature.

Transfer the slides to a jar with diluted Wash Buffer (see INSTRUCTIONS FOR USE, Section A.3) for 3 minutes at room temperature (20-25 °C).

Replace Wash Buffer and soak sections for another 3 minutes.

**NOTE:** The Pre-Treatment Solution is designed for a single use application only. Do not re-use.

**Step 2: Pepsin, ready-to-use**
Tap off excess buffer. Using lintless tissue (such as an absorbent wipe or gauze pad), carefully wipe around the specimen to remove any remaining liquid and to keep reagents within the prescribed area.

Apply 5-8 drops (250 µL) of cold (2-8 °C) Pepsin (Vial 2) to cover specimen. Always store Pepsin at 2-8 °C.

Incubate for 5-15 minutes at room temperature (20-25 °C). An incubation time of 10 minutes will be adequate for most specimens, but the optimal incubation time may depend on tissue fixation and/or thickness of specimen and should be determined by the user.

Tap off Pepsin and soak sections in the diluted Wash Buffer (see INSTRUCTIONS FOR USE, Section A.3) for 3 minutes at room temperature (20-25 °C).

Replace diluted Wash Buffer and soak sections for another 3 minutes.

Dehydrate tissue sections through a graded series of ethanol: 2 minutes in 70% ethanol, 2 minutes in 85% ethanol, and 2 minutes in 96% ethanol.

Allow tissue sections to air dry completely.

**Step 3: *HER2*/CEN-17 Probe Mix, ready-to-use**
The following step should be performed in a fume hood.

Apply 10 µL of *HER2*/CEN-17 Probe Mix (Vial 3) to the centre of the tissue section. Immediately place a 22 mm x 22 mm glass coverslip over the Probe Mix and allow it to spread evenly under the coverslip. Avoid air bubbles. If air bubbles are observed, gently tap them away from the tissue using forceps.

Seal coverslip with Coverslip Sealant by ejecting the Sealant around the periphery of the coverslip. Allow the Coverslip Sealant to overlap the coverslip and the slide, thereby forming a seal around the coverslip. Make sure that the Coverslip Sealant covers the entire edge of the coverslip.

Place slides on a flat metal or stone surface (heating block or on a block in a hybridization oven) preheated to 82 (±2) °C. Denature for 5 minutes ensuring that the temperature of the block does not drop below 80 °C at any time.

Place slides in a preheated humidified hybridization chamber. Cover the chamber with a lid and incubate overnight (14-20 hours) at **45 (±2) °C**. Please note that a hybridization temperature of 37 °C is not suitable for use with the probes contained within this kit.

Instrumentation that allows for conditions similar to the ones described above may be used for denaturation and hybridization.

**DAY 2**

**Step 4: Stringent Wash**
Fill two staining jars, e.g. Coplin jars, with the diluted Stringent Wash Buffer (see INSTRUCTIONS FOR USE, Section A.2). A minimum volume of 100 mL or 15 mL per slide in each jar is recommended.

Place staining jars containing Stringent Wash Buffer in water bath. Heat water bath and the diluted Stringent Wash Buffer to 65 (±2) °C. Ensure that the temperature has stabilized. Cover jars with lids in order to stabilize the temperature and avoid evaporation. Measure temperature inside jars with a calibrated thermometer to ensure correct temperature. The Stringent Wash Buffer contains detergent and may become turbid at 65 °C; this will not affect performance.

Remove one of the jars from the water bath and place in fume hood. Using forceps or gloves, gently remove Coverslip Sealant as well as coverslip and place slides in the jar, one at a time. Do not place slides in Stringent Wash Buffer before removing coverslips.

As soon as all coverslips have been removed, transfer slides to the 65 (±2) °C jar in the water bath. Perform stringent wash for exactly 10 minutes at 65 (±2) °C.

Remove slides from the diluted Stringent Wash Buffer, and soak sections in diluted Wash Buffer for 3 minutes at room temperature (20-25 °C).

Change diluted Wash Buffer and soak sections for another 3 minutes.

Dehydrate tissue sections through a graded series of ethanol: 2 minutes in 70% ethanol, 2 minutes in 85% ethanol, and 2 minutes in 96% ethanol.

Allow tissue sections to dry completely.

**Step 5: Mounting**
Apply 15 µL of Fluorescence Mounting Medium containing DAPI (Vial 5) to the target area of the slide and apply a glass coverslip.

**NOTE**: Slides may be read after 15 minutes or within 7 days after mounting. However, fading occurs if slides are exposed to light or high temperatures. To minimize fading, store slides in the dark at 2-8 °C.

## Quality Control

1. Signals must be bright, distinct and easy to evaluate.
2. Normal cells allow for an internal control of the staining run.
   - Normal cells should have 1-2 clearly visible green signals indicating that the CEN-17 PNA Probe has successfully hybridized to the centromeric region of chromosome 17.
   - Normal cells should also have 1-2 clearly visible red signals indicating that the *HER2* DNA Probe has successfully hybridized to the *HER2* amplicon.
   - Due to tissue sectioning, some normal cells will have less than the expected 2 signals of each colour.
   - Failure to detect signals in normal cells indicates assay failure, and results should be considered invalid.
3. Nuclear morphology must be intact when evaluated using a DAPI filter. Numerous ghost-like cells and a general poor nuclear morphology indicate over-digestion of the specimen, resulting in loss or fragmentation of signals. Such specimens should be considered invalid.
4. Differences in tissue fixation, processing, and embedding in the user's laboratory may produce variability in results, necessitating regular evaluation of in-house controls.

## Interpretation of Staining

**Assessable tissue**
Only specimens from patients with invasive carcinoma should be tested. In cases with carcinoma in situ and invasive carcinoma in the same specimen, only the invasive component should be scored. Avoid areas of necrosis and areas where the nuclear borders are ambiguous. Do not include nuclei that require subjective judgement. Skip nuclei with weak signal intensity and non-specific or high background.

**Signal enumeration:** Locate the tumour within the context of the H&E stained slide and evaluate the same area on the FISH stained slide. Scan several areas of tumour cells to account for possible heterogeneity. Select an area having good nuclei distribution. Begin analysis in the upper left quadrant of the selected area and, scanning from left to right, count the number of signals within the nuclear boundary of each evaluated nucleus according to the guidelines below (see also Appendix 3).

- Focus up and down to find all of the signals in the individual nucleus.
- Count two signals that are the same size and separated by a distance equal to or less than the diameter of the signal as only one signal.
- In nuclei with high levels of *HER2* gene amplification, the *HER2* signals may be positioned very close to each other forming a cluster of signals. In these cases the number of *HER2* signals cannot be counted, but must be estimated. Special attention must be paid to the green signals,

signals, as clusters of *HER2* signals can cover the green signals making them impossible to see. In case of doubt, please check the green signals using a specific FITC filter.

Do not score nuclei without signals or with signals of only one colour. Score only those nuclei with one or more FISH signals of each colour.

### Signal counting guide

| | | |
|---|---|---|
| 1 |  | Do not count. Nuclei are overlapping, not all areas of nuclei are visible |
| 2 |  | Two green signals, do not score nuclei with signals of only one color |
| 3 |  | Count as 3 green and 12 red signals (cluster estimation) |
| 4 |  | Count as 1 green and 1 red signal. Two signals of the same size and separated by a distance equal to or less than the diameter of one signal are counted as one |
| 5 |  | Do not count (over-digested nuclei) |
| 6 |  | Count as 2 green and 3 red signals. Two signals of the same size and separated by a distance equal to or less than the diameter of one signal are counted as one |
| 7 |  | Count as 1 green and 5 red signals |
| 8 |  | Count as 3 green (1 green out of focus) and 3 red signals |
| 9 |  | Cluster of red signals hiding green signals, check the green signals with a specific FITC filter, or do not count |

Record counts in a table as shown in Appendix 2.

Count 20 nuclei per tissue specimen, when possible from distinct tumor areas (19).

Calculate the *HER2*/CEN-17 ratio by dividing the total number of red *HER2* signals by the total number of green CEN-17 signals.

Specimens with a *HER2*/CEN-17 ratio above or equal to 2 should be considered *HER2* gene amplified (5, 17-19).

Results at or near the cut-off (1.8-2.2) should be interpreted with caution.

If the ratio is borderline (1.8-2.2), count an additional 20 nuclei and recalculate the ratio for the 40 nuclei.

In case of doubt, the specimen slide should be re-scored. For borderline cases a consultation between the pathologist and the treating physician is warranted.

## Limitations

1. FISH is a multi-step process that requires specialized training in the selection of the appropriate reagents, as well as in tissue selection, fixation, and processing, preparation of the FISH slide, and interpretation of the staining results.
2. FISH results are dependent on the handling and processing of the tissue prior to staining. Improper fixation, washing, drying, heating, sectioning, or contamination with other tissues or fluids may influence on probe hybridization. Inconsistent results may be due to variations in fixation and embedding methods, or to inherent irregularities within the tissue.

3. For optimal and reproducible results, the tissue slides must be deparaffinized completely. The paraffin removal needs to be completed at the beginning of the staining process. (See INSTRUCTIONS FOR USE, section B.2).
4. Only temperature-calibrated water bath, heating block, and hybridization oven should be used. Use of other types of equipment may result in evaporation of *HER2*/CEN-17 Probe Mix during hybridization and must be validated by user.

## Performance Characteristics

**Hybridization efficacy**
Hybridization efficacy of the *HER2* FISH pharmDx™ Kit was investigated at a routine pathology laboratory. 126 formalin-fixed, paraffin-embedded tissue sections were tested using the recommended procedure. Out of the 126 specimens, 124 could be scored according to the product guidelines, while 2 specimens could not be scored owing to technical reasons. Thus, the hybridization efficacy was 124/126 = 98% (20).

**Analytical sensitivity**
The sensitivity of the *HER2*/CEN-17 Probe Mix was investigated using 1 cell line with and 2 cell lines without amplification of the *HER2* gene. The ratio between the number of *HER2* signals and CEN-17 signals was calculated based on a counting of 60 nuclei per cell line.

The amplified cell line was scored as amplified with an average ratio of 3.21, while the 2 non-amplified cell lines were scored as non-amplified with average ratios of 0.96 and 1.17.

Furthermore, the *HER2*/CEN-17 ratios of 5 *HER2* gene non-amplified tissue sections were determined with the *HER2* FISH pharmDx™ Kit. Each tissue section was scored by 3 independent technicians. Results are presented in Table 1.

Table 1. HER2/CEN-17 ratios in 5 non-amplified tissues scored by 3 independent technicians

| | Tissue 1 | Tissue 2 | Tissue 3 | Tissue 4 | Tissue 5 |
|---|---|---|---|---|---|
| **Technician 1** | 0.95 | 1.01 | 1.04 | 1.21 | 1.15 |
| **Technician 2** | 1.05 | 1.00 | 0.99 | 1.11 | 1.09 |
| **Technician 3** | 1.05 | 1.00 | 0.92 | 1.14 | 1.06 |
| **Mean ratio** | 1.02 | 1.00 | 0.98 | 1.15 | 1.10 |
| **CV%** | 6 | 1 | 6 | 4 | 4 |

CV: Coefficient of variation

The study confirmed all 5 tissue sections to be non-amplified with a mean *HER2*/CEN-17 ratio close to 1.0.

**Analytical specificity**
The *HER2* DNA probes in the *HER2*/CEN-17 Probe Mix have been end-sequenced and mapped to confirm a total coverage of 218 kb including the *HER2* gene.

The CEN-17 PNA probes in the *HER2*/CEN-17 Probe Mix have been tested individually and in combination to confirm their specific hybridization to the centromeric region of chromosome 17.

To exclude cross-hybridization to chromosomes other than chromosome 17, studies were performed on metaphase spreads according to standard DakoCytomation QC procedures. A total of 250 metaphase spreads were evaluated for specific hybridization of the *HER2* DNA and CEN-17 PNA probe mixes. In all 250 cases the hybridization was specific for chromosome 17. No cross-hybridization to loci on other chromosomes was observed in any of the 250 cases.

**Testing of normal tissue**
To establish a range of expected results for normal tissue, a study that measured the distribution of HER2/CEN-17 ratios among normal breast specimens was conducted. In a sample set of 21 normal breast tissue specimens, the average ratio was found to be 1.06 with a

a 95% confidence interval of 1.02-1.10. The standard deviation was 0.09.

**Robustness studies**

The robustness of the *HER2* FISH pharmDx™ assay was tested by varying pre-treatment time and temperature, pepsin incubation time, denaturation temperature, hybridization time and temperature, and stringent wash time and temperature.

No significant difference in results was observed at the following experimental conditions:

- Pretreatment at 7, 10 and 13 minutes combined with each of the temperatures 89, 92 and 95-99 °C.
- Pepsin incubation times of 2, 5, 10, 15 and 18 minutes.
- Denaturation temperatures of 72, 77, 82, 87 and 92 °C.
- Hybridization time of 17 hours combined with each of the temperatures 40, 45 and 50 °C.
- Hybridization times of 10, 12 and 14 hours at a temperature of 45 °C.

The stringent wash was tested for 10 minutes at 60, 65 and 70 °C. Additionally, the stringent wash was tested for 5, 10 and 15 minutes at 65 °C. Stringent wash for 10 minutes at 70 °C, and stringent wash for 15 minutes at 65 °C resulted in loss of signals, whereas no significant difference in results was observed at the other time/temperature combinations. Furthermore the following dilutions of the Stringent Wash Buffer were tested: 1:10, 1:15, 1:20, 1:30 and 1:40. A 1:40 dilution of the Stringent Wash Buffer resulted in loss of signals, whereas no significant difference in results was observed for the other dilutions.

**Repeatability**

The repeatability of the *HER2*/CEN-17 ratio was investigated with the *HER2* FISH pharmDx™ Kit using consecutive sections of normal breast tissue and breast carcinoma. The coefficient of variation for normal breast tissue was found to be 6% and 4% for breast carcinoma.

A total of 10 consecutive sections of breast cancer tissue with different thickness (duplicates of 3, 4, 5, 6, and 7 µm) were tested with the *HER2* FISH pharmDx™ Kit. The coefficient of variation of the *HER2*/CEN-17 ratio in this study was found to be 12%, i.e. higher than for tissue sections of equal thickness.

**Reproducibility**

The *HER2* FISH pharmDx™ Kit was tested for lot-to-lot, day-to-day and observer-to-observer reproducibility using 3 different formalin-fixed and paraffin-embedded cell lines (the non-amplified MDA-231 and MDA-175, and the *HER2* gene amplified SKBR3). For these three studies, 30 nuclei were counted per specimen. The greatest *HER2*/CEN-17 ratio variation (8%) was found in the observer-to-observer study on the amplified cell line. This might be expected and possibly reflects a certain subjectivity in signal interpretation and enumeration. Results expressed as mean ratio, standard deviation, and coefficient of variation are presented in Tables 2-4.

Table 2. Lot-to-lot reproducibility. *HER2*/CEN-17 ratio measured for 3 different lots of *HER2* FISH pharmDx™ Kit

| Cell line | *HER2*/CEN-17 ratio | Kit lot 1 | Kit lot 2 | Kit lot 3 | Total |
|---|---|---|---|---|---|
| **MDA-231** | Mean | 1.06 | 1.04 | 1.07 | 1.06 |
| | SD | 0.04 | 0.04 | 0.05 | 0.04 |
| | CV% | 4 | 4 | 4 | 4 |
| | N | 5 | 5 | 5 | 15 |
| **MDA-175** | Mean | 1.23 | 1.20 | 1.16 | 1.20 |
| | SD | 0.02 | 0.05 | 0.07 | 0.06 |
| | CV% | 1 | 4 | 6 | 5 |
| | N | 5 | 5 | 5 | 15 |
| **SKBR3** | Mean | 3.99 | 3.77 | 3.82 | 3.86 |
| | SD | 0.18 | 0.19 | 0.29 | 0.23 |
| | CV% | 5 | 5 | 8 | 6 |
| | N | 5 | 5 | 5 | 15 |

SD: Standard deviation CV: Coefficient of variation N: number of slides

Table 3. Day-to-day reproducibility. *HER2*/CEN-17 ratio measured on 4 different days

| Cell line | *HER2*/CEN-17 ratio | Day 1 | Day 2 | Day 3 | Day 4 | Total |
|---|---|---|---|---|---|---|
| **MDA-231** | Mean | 1.04 | 1.03 | 1.05 | 0.99 | 1.03 |
| | SD | 0.05 | 0.02 | 0.03 | 0.01 | 0.04 |
| | CV% | 5 | 2 | 3 | 1 | 3 |
| | N | 5 | 5 | 5 | 5 | 20 |
| **MDA-175** | Mean | 1.26 | 1.17 | 1.25 | 1.16 | 1.21 |
| | SD | 0.06 | 0.04 | 0.07 | 0.04 | 0.07 |
| | CV% | 5 | 4 | 6 | 3 | 6 |
| | N | 5 | 5 | 5 | 5 | 20 |
| **SKBR3** | Mean | 4.30 | 4.59 | 4.56 | 4.09 | 4.39 |
| | SD | 0.39 | 0.32 | 0.15 | 0.08 | 0.32 |
| | CV% | 9 | 7 | 3 | 2 | 7 |
| | N | 5 | 5 | 5 | 5 | 20 |

SD: Standard deviation CV: Coefficient of variation N: Number of slides

Table 4. Observer-to-observer reproducibility. *HER2*/CEN-17 ratio measured by 3 different observers

| Cell line | *HER2*/CEN-17 ratio | Obs. 1 | Obs. 2 | Obs. 3 | Total |
|---|---|---|---|---|---|
| **MDA-231** | Mean | 1.03 | 1.03 | 1.09 | 1.05 |
| | SD | 0.02 | 0.08 | 0.05 | 0.06 |
| | CV% | 2 | 8 | 5 | 6 |
| | N | 5 | 5 | 5 | 15 |
| **MDA-175** | Mean | 1.17 | 1.15 | 1.11 | 1.14 |
| | SD | 0.02 | 0.05 | 0.11 | 0.07 |
| | CV% | 2 | 4 | 10 | 6 |
| | N | 5 | 5 | 5 | 15 |
| **SKBR3** | Mean | 4.03 | 3.57 | 3.63 | 3.74 |
| | SD | 0.18 | 0.19 | 0.24 | 0.29 |
| | CV | 5 | 5 | 7 | 8 |
| | N | 5 | 5 | 5 | 15 |

SD: Standard Deviation CV: Coefficient of Variation N. Number of slides

Interobserver Study

A second Inter-observer study was conducted on archived breast cancer tissue specimens selected to reflect a range of *HER2* amplification levels. Three observers counted events in 20 nuclei for each of 27 specimens. Concordance between observers with regard to amplification/non-amplification status was 100% in all cases

**Assay portability**

To assess interlaboratory reproducibility (assay portability) a blinded, randomized, comparative study of *HER2*/CEN-17 ratios measured in 4 formalin-fixed, paraffin-embedded breast cancer specimens was conducted involving 5 different study sites. The 4 specimens included in the study represented varying levels of *HER2* gene amplification and were selected to reflect a natural range of amplification rates, including a non-amplified (measured ratio 0.9-1.2), an altered but non-amplified (measured ratio 1.4-1.7), a low-level amplified (measured ratio 3.0-4.0), and a high level amplified (measured ratio 5.0-8.0). Each site stained and interpreted the 4 specimens in 3 separate runs (total of 12 slides). A provided control was also included in each run.

When stratifying results as either *HER2* gene amplification positive or negative (cut-off ratio = 2.0), there was complete agreement between the 5 sites, please see Table 5.

Table 5. Summary of portability results

| Specimen | *HER2* negative | *HER2* positive |
|---|---|---|
| **Non-amplified** | 15 | 0 |
| **Altered but non-amplified** | 15 | 0 |
| **Low-level amplification** | 0 | 15 |
| **High-level amplification** | 0 | 15 |

A day-to-day variation of 10% was found in the 5 laboratories for the *HER2*/CEN-17 ratio.

A site-to-site variation for the *HER2*/CEN-17 ratio of approx. 10-15% was observed for the non-amplified cases and the cases with ratios close to the cut off. This figure is consistent with findings reported in the literature. A higher variation (25%) was observed for the highly amplified specimen; also this is in concordance with the literature, and this variation is not considered clinically relevant (17). As the scoring of highly amplified cases where signals are clustered cannot be based on counting, but must be based on an estimation of the number of signals, a high variation for such cases can be expected.

**Clinical utility**

Studies involving the *HER2* FISH pharmDx™ kit included comparison testing using the PathVision Kit as the reference method, and/or DakoCytomation HercepTest™. Results of *HER2* FISH testing are available for a total of 940 breast cancer specimens.

Comparison with PathVysion Kit test results

Three studies have been performed that compare the results of *HER2* FISH pharmDx™ testing to the results of PathVysion Kit testing. The studies were performed in geographically separate locations, and there was no overlap in the use of specimens. A total of 328 specimens have been tested.

An overview of the three studies is presented in Table 6.

Table 6. Summary data of FISH method comparison studies

| Study designation | Concordance study (Danish specimens) | Concordance study (Japanese specimens) | French study (21) |
|---|---|---|---|
| N | 190 | 52 | 86 |
| Concordance (95% confidence interval) | 93.68% (90.22% to 97.14%) | 96.15% | |
| Positive percent agreement (95% confidence interval) | 86% (77.34% to 95.08%) | 97% | |
| Negative percent agreement (95% confidence interval) | 97% (94.05% to 99.89%) | 96% | |

The following table summarizes the 12 discrepant test results between *HER2* FISH pharmDx™ test and PathVysion™ HER-2 Probe test for the Danish clinical specimens.

| *HER2* FISH(+)/PathVysion(-) | | | | *HER2* FISH(-)/PathVysion(+) | | | |
|---|---|---|---|---|---|---|---|
| ID | *HER2* FISH | PathVysion | HercepTest | ID | *HER2* FISH | PathVysion | HercepTest |
| 160 | 2.10* (1.82-2.51) | 1.51 (1.39-1.68) | 2 | 234 | 1.68 (1.38-1.83) | 2.02* (1.84-2.29) | 2 |
| 208 | 3.61 (2.95-4.73) | 1.62 (1.51-1.82) | 2 | 284 | 1.44 (1.07-1.83) | 2.21* (1.94-2.64) | 2 |
| 306 | 2.20* (1.79-2.24) | 1.33 (1.18-1.44) | 1 | 423 | 1.7 (1.52-1.95)5 | 2.15 (2.02-2.45) | 1 |
| 846 | 2.58 (2.06-3.50) | 1.51 (1.42-1.76) | 2 | 474 | 1.44 (1.16-1.83) | 2.55 (2.38-3.26) | 2 |
| | | | | 735 | 1.68 (1.40-1.99) | 2.03* (1.89-2.19) | 2 |
| | | | | 746 | 1.05 (0.96-1.18) | 4.53 (4.27-5.17) | 3 |
| | | | | 837 | 1.52 (1.48-1.79) | 2.15 (2.10-2.67) | 3 |
| | | | | 881 | 1.83* (1.15-2.69) | 2.68 (2.39-3.14) | 2 |

*CI of mean log ratios included 2.0

In this discrepancy analysis, logged ratios were used. The 95% confidence interval was calculated for the 60 logged ratios from the nuclei that were used to calculate the PathVysion ratio. For the 4 instances where *HER2* FISH was positive and PathVysion was negative, no interval included the critical value of 2. Of the eight instances where *HER2* FISH was negative and PathVysion was positive, the 95% CI of 3 (#234, 284 and 735) included the critical value of 2. Similarly, The 95% confidence interval was calculated for the logged ratios from the nuclei that were used to calculate the *HER2* FISH ratio. For the 4 instances where *HER2* FISH was positive and PathVysion was negative, 2 included the critical value of 2 (#160 and 306). Of the eight instances where *HER2* FISH was negative and PathVysion was positive, the 95% CI of 1 (#881) included the critical value of 2.

Comparison with HercepTest™ results

Four studies comparing HER2 FISH to HercepTest™ results have been completed. A total of 940 specimens have been compared, using 3+ HercepTest™ result as a positive IHC result. An overview of the three studies is presented in Table 7.

Table 7. Summary of DakoCytomation *HER2* FISH/IHC (HercepTest™) comparison studies

| **Study designation** | **Danish clinical specimens** | **Japanese specimens** | **French study (21)** | **Danish Pilot study (20)** |
|---|---|---|---|---|
| N | 682 | 52 | 86 | 120 |
| Concordance (95% confidence interval) | 93.11% (91.21% - 95.01%) | 96,15% | 87.21% | 93.33% |
| Positive percent agreement (95% confidence interval) | 91% (87.39% - 94.57%) | 96% | 87% | 84% |
| Negative percent agreement (95% confidence interval) | 94% (92.12% - 96.46%) | 96% | 87% | 97% |

More complete distribution data of HercepTest™ and *HER2* FISH test results for the Danish clinical specimens is presented in Table 8.

Table 8. Distribution of HER2 status by HercepTest™ and *HER2* FISH

| **HercepTest™ score** | **0** | **1** | **2** | **3** | **Total** |
|---|---|---|---|---|---|
| **N** | 221 | 267 | 84 | 248 | 820 |
| **%** | 27 | 33 | 10 | 30 | 100% |
| **HER2 FISH status** | | | | | |
| **Amplified** | 0 | 8 | 17 | 222 | 247 |
| **Non-amplified** | 106 | 245 | 62 | 22 | 435 |
| **Total FISH tested samples** | 106 | 253 | 79 | 244 | 682 |

Alternative counting methods

Alternative counting methods, including counting a fixed number of events, and conventional counting signals in a fixed number of cells, were evaluated. The alternative counting method implies that more nuclei are counted in cases near the cut off than in highly amplified cases. Statistical analysis using simulation studies on a subset of data with complete 60 cell evaluation for both the DakoCytomation HER2 FISH pharmDx™ kit and the PathVysion Kit showed that the alternative counting methods yielded nearly the same concordance, concordance=0.94 when as few as 20 events (minimum 7 nuclei) were counted, and concordance=0.97 when 30 or 60 events (minimum 7 nuclei) were counted. For the two last methods the real number of nuclei counted was less than or equal to 15 and 20, respectively, in 90% of the cases.

In addition, different methods of calculating the *HER2/CEN-17* ratio were evaluated and found to provide equivalent ratios whether using a subset with as few as 20 nuclei being counted. Equivalent ratios were obtained whether the ratio was calculated from the sum of the *HER2* and *CEN-17* counts or by using logarithmically transformed individual ratios that were then averaged.

## Troubleshooting

| Problem | Probable Cause | Suggested Action |
|---|---|---|
| 1. No signals or weak signals | 1a. Kit has been exposed to high temperatures during transport or storage | 1a. Check storage conditions. Ensure that dry ice was present when the consignment was received. Ensure that vials 2, 3 and 5 have been stored at maximum 2-8 °C, and that vials 3 and 5 have been stored in the dark. |
| | 1b. Microscope not functioning properly<br>- Inappropriate filter set<br>- Improper lamp<br>- Mercury lamp too old<br>- Dirty and/or cracked collector lenses<br>- Unsuitable immersion oil | 1b. Check the microscope and ensure that the used filters are suitable for use with the kit fluorochromes, and that the mercury lamp is correct and has not been used beyond expected lifetime. (see Appendix 3). In case of doubt, please contact your local microscope vendor. |
| | 1c. Faded signals | 1c. Avoid long microscopic examination and minimize exposure to strong light sources. |
| | 1d. Pre-treatment conditions incorrect | 1d. Ensure that the recommended pre-treatment temperature and time are used. |
| | 1e. Evaporation of Probe Mix during hybridization | 1e. Ensure sufficient humidity in the hybridization chamber |
| 2. No green signals | 2a. Stringent wash conditions incorrect | 2a. Ensure that the recommended stringent wash temperature and time are used, and that coverslips are removed before performing stringent wash |
| 3. No red signals | 3a. Pre-treatment conditions incorrect | 3a. Ensure that the recommended pre-treatment temperature and time are used |
| 4. Areas without signal | 4a. Probe volume too small | 4a. Ensure that the probe volume is large enough to cover the area under the coverslip |
| | 4b. Air bubbles caught during Probe Mix application or mounting | 4b. Avoid air bubbles. If observed, gently tap them away using forceps |

| Problem | Probable Cause | Suggested Action |
|---|---|---|
| 5. Excessive back-ground staining | 5a. Inappropriate tissue fixation | 5a Ensure that only formalin-fixed, paraffin-embedded tissue sections are used |
| | 5b. Paraffin incompletely removed | 5b. Follow the deparaffinization and rehydration procedures outlined in Section B.2 |
| | 5c. Stringent wash temperature too low | 5c. Ensure that the stringent wash temperature is 65 (±2) °C |
| | 5d. Prolonged exposure of hybridized section to strong light | 5d. Avoid long microscopic examination and minimize exposure to strong light |
| 6. Poor tissue morphology | 6a. Incorrect Pepsin treatment | 6a. Adhere to recommended Pepsin incubation times. See section B.3, step 2. Ensure that the Pepsin is handled at the correct temperature. See Section B.1 |
| | 6b. Incorrect pre-treatment conditions may result in unclear or cloudy appearance | 6b. Ensure that the recommended pre-treatment temperature and time are used |
| | 6c. Too long Pepsin treatment or very thin section thickness may cause ghost cells or donut cells to appear. | 6c. Shorten the Pepsin incubation time. See section B.3, step 2. Ensure that the section thickness is 4-6 µm. |

**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 our Technical Services for further assistance.

# Appendix 1

***HER2* FISH pharmDx™ Kit, code No. K 5331**
Protocol Checklist

**Staining Run Log ID:**_____

Date (Day 1) of the run: _____

*HER2* FISH pharmDx™ Kit, K 5331 Lot: _____

Specimen ID: _____

Equipment ID: _____

Date of dilution/expiration of the 1 x Wash Buffer (Vial 6 diluted 1:20):_____/_____

| | |
|---|---|
| Tissue fixed in neutral buffered formalin | **Yes** ☐ **No** ☐ |

**DAY 1**

| | |
|---|---|
| **Step 1: Pre-Treatment** | |
| Date of dilution/expiration of the Pre-Treatment Solution (Vial 1 diluted 1:20) | / |
| Measured temperature of Pre-Treatment Solution (95-99 °C) | °C |
| Pre-treatment (10 minutes), and cooling (15 minutes) | |
| Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes) | |
| **Step 2: Pepsin** | |
| Duration of Pepsin (Vial 2) treatment (5-15 minutes) | Minutes |
| Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes) | |
| Dehydrate slides (3 x 2 minutes) in graded series of ethanol and air dry | |
| **Step 3: *HER2*/CEN-17 Probe Mix** | |
| Apply Probe Mix (Vial 3), coverslip and seal with Coverslip Sealant | |
| Measured denaturation temperature (82 ±2 °C) | °C |
| Denaturation for 5 minutes | |
| Measured hybridization temperature (45 ±2 °C) | °C |
| Hybridization overnight (protect from light) | |

**DAY 2**

| | |
|---|---|
| **Step 4: Stringent Wash** | |
| Date of dilution/expiration of the Stringent Wash Buffer (Vial 4 diluted 1:20) | / |
| Measured temperature of Stringent Wash Buffer (65 ±2 °C) | °C |
| Stringent wash (10 minutes) after removing the coverslips | |
| Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes) | |
| Dehydrate slides (3 x 2 minutes) in graded series of ethanol and air dry | |
| **Step 5: Mounting** | |
| Apply 15 µL of Fluorescence Mounting Medium (Vial 5) and coverslip | |

Comments:_____

_____

Date and signature, Technician:_____

# Appendix 2

## *HER2* FISH pharmDx™ Kit, code No. K 5331

**Scoring Scheme**

Staining Run Log ID: _____

Date (Day 1) of the run: _____

*HER2* FISH pharmDx™ Kit, K 5331 Lot: _____ Specimen ID: _____

| Count signals in 20 nuclei | | | | | |
|---|---|---|---|---|---|
| Nucleus No. | *HER2* score (red) | CEN-17 score (green) | Nucleus No. | *HER2* score (red) | CEN-17 score (green) |
| 1 | | | 11 | | |
| 2 | | | 12 | | |
| 3 | | | 13 | | |
| 4 | | | 14 | | |
| 5 | | | 15 | | |
| 6 | | | 16 | | |
| 7 | | | 17 | | |
| 8 | | | 18 | | |
| 9 | | | 19 | | |
| 10 | | | 20 | | |
| Total (1-10) | | | Total (11-20) | | |

For determination of the *HER2*/CEN-17 ratio, count the number of *HER2* signals and the number of CEN-17 signals in the same 20 nuclei and divide the total number of *HER2* signals by the total number of CEN-17 signals. If the *HER2*/CEN-17 ratio is borderline (1.8-2.2), count an additional 20 nuclei and recalculate the ratio.

A ratio at or near the cut-off (1.8-2.2) should be interpreted with caution (see counting guide).

| | *HER2* | CEN-17 | *HER2/CEN-17 ratio* |
|---|---|---|---|
| Total score (1-20) | | | |

☐ Ratio < 2: *HER2* gene amplification was not observed

☐ Ratio > = 2: *HER2* gene amplification was observed

Date and signature, Technician: _____

Date and signature, Pathologist: _____

*For scoring guidelines: see Interpretation of Staining.*

(placeholder)

# Appendix 3

***HER2* FISH pharmDx™ Kit, code No. K 5331**

**Fluorescence Microscope Specifications**

**DakoCytomation recommends the following equipment for use with the *HER2* FISH pharmDx™ Kit, K 5331:**

1. **Microscope type**
   - Epifluorescence microscope.
2. **Lamp**
   - 100 watt mercury lamp (keep record of burning time).
3. **Objectives**
   - For screening of the tissue, fluorescence dry 10X or fluorescence oil immersion 16X objectives are applicable.
   - For high power magnification and scoring of signals, only fluorescence oil immersion objectives, e.g. 100X are recommended.
4. **Filters**

Filters are individually designed for specific fluorochromes and must be chosen accordingly. DakoCytomation recommends the use of a specific DAPI filter in combination with a high quality Texas Red/FITC double filter.

- DAPI filter, e.g. Chroma filter # 31000.
- Texas Red/FITC double filter, e.g. Omega Optical filter # XF53 or Chroma filter # 51006.
- Texas Red and FITC single filters can be used for confirmation.

| Fluorochrome | Excitation Wavelength | Emission Wavelength |
|---|---|---|
| FITC | 495 nm | 520 nm |
| Texas Red | 596 nm | 615 nm |

Filters are specific to each microscope type and the use of appropriate filters is crucial for the interpretation. If you want detailed information, please contact your microscope provider or your DakoCytomation representative.

5. **Oil**
   - Non-fluorescing oil.

**Precautions**

- A 50 watt mercury lamp is not recommended.
- Rhodamine filters cannot be used.
- Triple filters are not recommended.

A non-optimized microscope may cause problems when reading the fluorescent signals. It is important that the light source has not expired and that it is properly aligned and focused.

Customers should monitor and follow the manufacturer's recommendations for the mercury lamp. The microscope should be maintained and the mercury lamp should be in alignment prior to interpreting results.

An effort should be made to expose the sample to as little of the excitation light as possible in order to minimize fading of the fluorescence.

We recommend that you discuss the set-up of your particular microscope with the manufacturer before starting the fluorescence in situ hybridization, or refer to the literature.

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**Explanation of symbols**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| REF | Catalogue number | -20°C / 8°C | Temperature limitation | LOT | Batch code | | Toxic |
| IVD | In vitro diagnostic medical device | | Keep away from sunlight (consult storage section) | | Use by | | Extremely flammable |
| | Consult instructions for use | Σ | Contains sufficient for <n> tests | | Manufacturer | | Dangerous for the environment |