**HER2 IQFISH pharmDx**

Code K5731

11th edition

**HER2 IQFISH pharmDx** is a direct fluorescence in situ hybridization (FISH) assay designed to quantitatively determine **HER2** gene amplification in formalin-fixed, paraffin-embedded (FFPE) breast cancer tissue specimens and FFPE specimens from patients with metastatic gastric or gastroesophageal junction adenocarcinoma.

**HER2 IQFISH pharmDx** is indicated as an aid in the assessment of breast and gastric cancer patients for whom Herceptin® (trastuzumab) treatment is being considered and for breast cancer patients for whom PERJETA™ (pertuzumab) or KADCYLA™ (ado-trastuzumab emtansine) treatment is being considered (see Herceptin®, PERJETA™ and KADCYLA™ package inserts).

For breast cancer patients, results from the **HER2 IQFISH pharmDx** 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.
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Intended Use

HER2 IQFISH pharmDx is a direct fluorescence in situ hybridization (FISH) assay designed to quantitatively determine HER2 gene amplification in formalin-fixed, paraffin-embedded (FFPE) breast cancer tissue specimens and FFPE specimens from patients with metastatic gastric or gastroesophageal junction adenocarcinoma.

HER2 IQFISH pharmDx is indicated as an aid in the assessment of breast and gastric cancer patients for whom Herceptin® (trastuzumab) treatment is being considered and for breast cancer patients for whom PERJETA™ (pertuzumab) or KADCYLA™ (ado-trastuzumab emtansine) treatment is being considered (see Herceptin®, PERJETA™ and KADCYLA™ package inserts).

For breast cancer patients, results from the HER2 IQFISH pharmDx are intended for use as an adjunct to the clinicopathologic information currently used for estimating prognosis in stage II, node-positive breast cancer patients.

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

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

For breast cancer application, please refer to pages 5-38.
For gastric cancer application, please refer to pages 39-69.

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

The human HER2 gene (also known as ERBB2 or NEU) is located on chromosome 17 and encodes the HER2 protein or p185HER2. The HER2 protein is a membrane receptor tyrosine kinase with homology to the epidermal growth factor receptor (EGFR or HER1) (1-2). 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 tumor progression (3-8). HER2 gene amplification generally leads to overexpression of the HER2 protein on the surface of breast cancer cells (9).

Amplification of the HER2 gene and/or overexpression of its protein have been demonstrated in 20-25% of breast cancers (10). 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 (11).

Demonstration of high HER2 protein overexpression or HER2 gene amplification is essential for initiating therapy with Herceptin®, a monoclonal antibody to HER2 protein. Clinical studies have shown that patients whose tumors have high HER2 protein overexpression and/or amplification of the HER2 gene benefit most from Herceptin® (12).

Pertuzumab is a recombinant, humanized monoclonal antibody that binds to subdomain II of the extracellular part of the HER2 protein thereby blocking its ability to form heterodimers with other members of the HER family including HER1 (EGFR), HER3, and HER4 (13-15)). PERJETA™ (pertuzumab) has shown to be effective and safe in treatment of breast cancer patients with HER2 protein overexpression. During clinical studies of pertuzumab, HER2 overexpression was demonstrated directly by IHC or indirectly evidenced through correlation of HER2 gene amplification to protein overexpression as demonstrated by FISH. However, in the randomized trial, data were available for a limited number of patients (8/808) for whom the FISH results were positive but the IHC results were negative (0, 1+) (16-17).

Ado-trastuzumab emtansine is a novel antibody–drug conjugate specifically designed for the treatment of HER2-positive cancer. It is composed of the potent cytotoxic agent DM1 (a thiol-containing maytansinoid anti-microtubule agent) conjugated to trastuzumab via a linker molecule. Ado-trastuzumab emtansine binds to HER2 with an affinity similar to that of trastuzumab; such binding is required for its anti-tumor activity. It is hypothesized that after binding to HER2, ado-trastuzumab emtansine undergoes receptor-mediated internalization, followed by intracellular release of DM1 and subsequent cytotoxicity (18). A number of clinical studies have shown that ado-trastuzumab emtansine is effective and safe in treatment of HER2-positive breast cancer patients (19-22).

Principle of Procedure - Breast

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

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, at 37 °C for 3-5 minutes or by immersing the slides into Pepsin solution at 37 °C for 20-30 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) (23) 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. 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), tumor 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 - Breast

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 2A, 10 µL per slide of Vial 3, and 15 µL per slide of Vial 5). The solutions in Vial 3 and Vial 5 are viscous and may have to be centrifuged shortly in a microcentrifuge in order to be able to collect all of the provided reagent.

The kit provides materials sufficient for 10 individual staining runs (four separate runs, when using the pepsin immersing method).

**HER2 IQFISH pharmDx** 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 **HER2 IQFISH pharmDx**.

**Vial 1**
**PRE-TREATMENT SOLUTION (20x)**
Pre-Treatment Solution (20x)
150 mL, concentrated 20x
MES (2-[N-morpholino]ethanesulphonic acid) buffer.

**Vial 2A**
**PEPSIN**
Pepsin
4 x 6.0 mL, ready-to-use
Pepsin solution, pH 2.0; contains stabilizer and an antimicrobial agent.

**Vial 2B**
**PEPSIN DILUENT (10x)**
Pepsin Diluent (10x)
24 mL, concentrated 10x
Dilution buffer, pH 2.0; contains an antimicrobial agent.

**Vial 3**
**HER2/CEN-17 IQISH PROBE MIX**
**HER2/CEN-17 IQISH Probe Mix**
0.2 mL, ready-to-use
Mix of Texas Red-labelled **HER2** DNA probes and fluorescein-labelled CEN-17 PNA probes; supplied in IQISH hybridization buffer.

**Vial 4**
**STRINGENT WASH BUFFER (20x)**
Stringent Wash Buffer (20x)
150 mL, concentrated 20x
SSC (saline-sodium citrate) buffer with detergent (Tween-20).

**Vial 5**
**FLUORESCENCE MOUNTING MEDIUM**
Fluorescence Mounting Medium
0.4 mL, ready-to-use
Fluorescence mounting medium with 500 µg/L DAPI (4',6-diamidine-2-phenylindole).
**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
- Dako Hybridizer (Code S2450)*
- Heating block or hybridization oven for denaturation (66 (±1) °C)*
- Humid hybridization chamber*
- Microcentrifuge (tabletop centrifuge for spinning down probe and mounting media)
- Slides, Dako Silanized Slides, Code S3003, or poly-L-lysine-coated slides (see Specimen Preparation)
- Staining jars or baths
- Timer (capable of 2-15 minute intervals)
- Vortex mixer
- Water bath with lid (capable of maintaining 37(±2) °C, 63 (±2) °C and from 95 °C to 99 °C)

*Microwave oven with sensing capability if pre-treatment is performed using microwave oven (see Section B.3. Staining protocol. Step 1: Pre-treatment, Method B)*

**NOTE:** Kit accessory reagents: Pre-Treatment Solution, Pepsin, Pepsin Diluent, Stringent Wash Buffer, Fluorescence Mounting Medium, Wash Buffer, and Coverslip Sealant can be substituted by identical Dako retail reagents in Dako Histology FISH Accessory Kit, Code K5799.
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 as light source should be used. Other light sources are not recommended with these filters.

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

Precautions - Breast

1. For in vitro diagnostic use.
2. For professional users.
3. Vial 1, Pre-Treatment Solution (20x), does not require hazard labeling. Safety Data Sheets (SDS) is available for professional users on request.
4. Vial 2A, Pepsin, contains 5-10% propan-2-ol, 0.1-1% pepsin A, and <0.1% 3(2H)-Isothiazolone, 5-chloro-2-methyl-, mixt. with 2-methyl-3(2H)-isothiazolone. Vial 2A is labeled:

   ![Danger]

   **Danger**

   H314 Causes severe skin burns and eye damage.
   H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled.
   H317 May cause an allergic skin reaction.
   H373 May cause damage to organs through prolonged or repeated exposure.
   P280 Wear protective gloves. Wear eye or face protection. Wear protective clothing.
   P285 In case of inadequate ventilation wear respiratory protection.
   P260 Do not breathe vapor.
   P264 Wash hands thoroughly after handling.
   P272 Contaminated work clothing should not be allowed out of the workplace.
   P314 Get medical attention if you feel unwell.
   P304 + P340 + P310 IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing. Immediately call a POISON CENTER or physician.
   P342 + P311 If experiencing respiratory symptoms: Call a POISON CENTER or physician.
   P301 + P310 + P330 + P331 IF SWALLOWED: Immediately call a POISON CENTER or physician. Rinse mouth. Do NOT induce vomiting.
   P303 + P361 + P353 + P363 + P310 IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water or shower. Wash contaminated clothing before reuse. Immediately call a POISON CENTER or physician.
   P302 + P352 IF ON SKIN: Wash with plenty of soap and water.
   P333 + P313 If skin irritation or rash occurs: Get medical attention.
   P305 + P351 + P338 + P310 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER or physician.
   P405 Store locked up.
   P501 Dispose of contents and container in accordance with all local,
Regional, national and international regulations.

5. Vial 2B, Pepsin Diluent (10x), contains 30-60% propan-2-ol, and 5-10% 2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride. Vial 2B is labeled:

**Danger**

- H325 Highly flammable liquid and vapor.
- H319 Causes serious eye irritation.
- H335 May cause respiratory irritation.
- H336 May cause drowsiness and dizziness.
- P373 May cause damage to organs through prolonged or repeated exposure.
- P280 Wear protective gloves. Wear eye or face protection.
- P210 Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.
- P241 Use explosion-proof electrical, ventilating, lighting and all material-handling equipment.
- P242 Use only non-sparking tools.
- P243 Take precautionary measures against static discharge.
- P271 Keep container tightly closed.
- P260 Do not breathe vapor.
- P264 Wash hands thoroughly after handling.
- P314 Get medical attention if you feel unwell.
- P304 + P340 + P312 IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing. Call a POISON CENTER or physician if you feel unwell.
- P303 + P361 + P353 IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water or shower.
- P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
- P337 + P313 If eye irritation persists: Get medical attention.
- P405 Store locked up.
- P403 Store in a well-ventilated place.
- P235 Keep cool.
- P501 Dispose of contents and container in accordance with all local, regional, national and international regulations.

6. Vial 3, HER2/CEN-17 IQISH Probe Mix, contains 10-30% ethylene carbonate, and 1-5% sodium chloride. Vial 3 is labeled:

**Warning**

- H319 Causes serious eye irritation.
- P280 Wear eye or face protection.
- P264 Wash hands thoroughly after handling.
- P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
- P337 + P313 If eye irritation persists: Get medical attention.

7. Vial 4, Stringent Wash Buffer (20x), contains 10-30% sodium chloride, and 10-30% 2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride. Vial 4 is labeled:
Breast Cancer

![Warning]

**Warning**

H319 Causes serious eye irritation.
H315 Causes skin irritation.
P280 Wear protective gloves. Wear eye or face protection.
P264 Wash hands thoroughly after handling.
P302 + P352 + P362-2 IF ON SKIN: Wash with plenty of soap and water. Take off contaminated clothing. Wash contaminated clothing before reuse.
P332 + P313 If skin irritation occurs: Get medical attention.
P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337 + P313 If eye irritation persists: Get medical attention.

8. Vial 6, Wash Buffer (20x), contains 10-30% sodium chloride, and 10-30% trometamol. Vial 6 is labeled:

![Warning]

**Warning**

H319 Causes serious eye irritation.
H315 Causes skin irritation.
P280 Wear protective gloves. Wear eye or face protection.
P264 Wash hands thoroughly after handling.
P302 + P352 + P362-2 IF ON SKIN: Wash with plenty of soap and water. Take off contaminated clothing. Wash contaminated clothing before reuse.
P332 + P313 If skin irritation occurs: Get medical attention.
P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337 + P313 If eye irritation persists: Get medical attention.

9. Coverslip Sealant contains 60-100% naphtha (petroleum), hydrotreated light, and is labeled:

![Danger]

**Danger**

H225 Highly flammable liquid and vapor.
H304 May be fatal if swallowed and enters airways.
P280 Wear protective gloves. Wear eye or face protection.
P210 Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.
P241 Use explosion-proof electrical, ventilating, lighting and all material-handling equipment.
P242 Use only non-sparking tools.
P243 Take precautionary measures against static discharge.
P233 Keep container tightly closed.
P301 + P310 + P331 IF SWALLOWED: Immediately call a POISON CENTER or physician. Do NOT induce vomiting.
P303 + P361 + P353 IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water or shower.
P405 Store locked up.
Breast Cancer

P403 Store in a well-ventilated place.
P235 Keep cool.
P501 Dispose of contents and container in accordance with all local, regional, national and international regulations.

10. 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 (24). 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.

11. Minimize microbial contamination of reagents to avoid erroneous results.

12. Incubation times and temperatures, or methods other than those specified, may give erroneous results.

13. Tissue fixation methods and thickness of specimen other than those specified may affect tissue morphology and/or signal intensity.

14. Avoid evaporation of HER2/CEN-17 Probe Mix during hybridization by ensuring sufficient humidity in the hybridization chamber.

15. Reagents have been optimally diluted. Further dilution may result in loss of performance.

16. Wear appropriate personal protective equipment to avoid contact with eyes and skin. Please refer to the Safety Data Sheet (SDS) for additional information.

17. Only clean staining jars should be used for the pepsin immersion method (Step 2, method C).

Storage - Breast
Store the HER2/CEN-17 IQISH Probe Mix (Vial 3) at -18 °C. All other reagents can be stored at 2-8 °C in the dark. All reagents tolerate frozen storage. Freezing and thawing the probe mix for up to 10 times does not affect performance.

Pepsin, HER2/CEN-17 IQISH Probe Mix, and Fluorescence Mounting Medium (Vials 2A, 3 and 5) may be affected adversely if exposed to heat. Do not leave these components at room temperature.

The HER2/CEN-17 IQISH 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 (25).

There are no obvious signs indicating 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 IQFISH pharmDx is suspected, contact Dako Technical Services immediately.

Specimen Preparation - Breast
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 (26).

Paraffin-embedded sections
Only tissue preserved in neutral buffered formalin and paraffin-embedded is 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) (26-27). 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 tumor presence should be prepared at the same time. A minimum of 2 serial sections is recommended, 1 section for tumor 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 Dako Silanized Slides, Code S3003, 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 - Breast

A. Reagent Preparation - Breast
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 20x) 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 20x) 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 20x) 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.

A.5 Pepsin Solution
A pepsin solution is only needed when using the pepsin immersing method (Method C).
Prepare pepsin solution as follows;
For a six slide capacity container prepare 60 mL pepsin solution:
Add 48 mL room temperature (20-25 °C) distilled or deionized water to the container.
Add 6 mL cold (2-8 °C) Pepsin Diluent (10x) (Vial 2B) to the container.
Add 6 mL cold (2-8 °C) Pepsin (Vial 2A) to the container.
Put lid on the container and equilibrate the pepsin solution to 37 (±2) °C in a water bath.

For a 24 slide capacity container prepare 240 mL pepsin solution:
Add 192 mL room temperature (20-25 °C) distilled or deionized water to the container.
Add 24 mL cold (2-8 °C) Pepsin Diluent (10x) (Vial 2B) to the container.
Add 24 mL cold (2-8 °C) Pepsin (Vial 2A) to the container.
Put lid on the container and equilibrate the pepsin solution to 37 (±2) °C in a water bath.

Equilibrated pepsin solution should be used within 5 hours.
B. Staining Procedure - Breast

B.1 Procedural notes

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

All reagents should be equilibrated to the relevant temperature prior to use as follows:

Vial 1: The diluted Pre-Treatment Solution should be equilibrated to 95-99 °C if water bath is used for pre-treatment (Section B.3. Staining protocol, Step 1: Pre-Treatment Method A). If microwave oven with sensing capability is used for pre-treatment (Section B.3. Staining protocol, Step 1: Pre-Treatment, Method B) the diluted Pre-Treatment Solution should be equilibrated to room temperature 20-25 °C.

Vial 2A: Pepsin should be applied at 2-8 °C (Section B.3, Staining protocol, Step 2 Method A and B) and kept cold continuously.

Vial 2B: Pepsin Diluent (10x) should be applied at 2-8 °C (Section B.3, Staining protocol, Step 2 Method C).

Vial 3: HER2/CEN-17 IQISH Probe Mix separates into two phases while stored at -18 °C. Prior to use of Vial 3 ensure that only one phase is present by equilibration to room temperature (20-25 °C) followed by mixing. Thaw Vial 3 at room temperature (20-25 °C) for a maximum of 30 minutes (protect from strong light), then thoroughly whirl the vial for 15 seconds at 2500 rpm using a vortex mixer. Store Vial 3 at -18 °C immediately after use.

Vial 4: The Diluted Stringent Wash Buffer; one jar should be equilibrated to room temperature, another jar should be equilibrated to 63 (±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 temperature 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

#### Step 1: Pre-Treatment

Pre-treatment can be performed either by using water bath as described in method A) or, alternatively, by use of microwave oven with sensing capability as described in method B).

**Method A) Pre-treatment using water bath:**

Fill staining jars, e.g. Coplin jars, with the diluted Pre-Treatment Solution (see INSTRUCTIONS FOR USE, Section A.1). Place staining jars containing diluted Pre-Treatment Solution in water bath. Heat water bath and the Pre-Treatment Solution to 95-99 ºC. 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 ºC.

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 ºC).

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.

**Method B) Pre-Treatment using microwave oven with sensing capability:**

Fill a plastic jar with diluted room temperature (20-25 ºC) Pre-Treatment Solution. Immerse the deparaffinized sections in Pre-Treatment Solution, cover the jar with a punctured lid and place it in the microwave oven. Select the boiling sensor function and a program that runs for 10 minutes after boiling temperature has been reached*.

Following the 10 minutes incubation take the jar with slides out of the oven, remove the lid and cool for 15 minutes at room temperature. Transfer the slides to a jar with diluted Wash Buffer and soak for 3 minutes at room temperature (20-25 ºC). Replace Wash Buffer and soak sections for another 3 minutes.

* The use of a microwave oven with a sensing capability means that the oven must include a sensor and programs which initially heat the Pre-Treatment Solution to the boiling point and subsequently maintain the required pre-treatment temperature (above 95 ºC) while counting down the preset time (10 ±1 minutes). Some microwave oven models with sensing capability may not include the possibility to freely set a count-down time. If the model only includes pre-set programs, be sure to select a program which maintain the required pre-treatment temperature (above 95 ºC) for at least 10 (±1) minutes and manually stop the program after 10 (±1) minutes.

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

#### Step 2: Pepsin, ready-to-use (RTU) or pepsin solution
Pepsin incubation can be performed by direct application of RTU pepsin drops to the slides either at room temperature (20-25 °C) (Method A) or at 37 °C (Method B). Alternatively, slides can be immersed into a pepsin solution and incubated at 37 (±2) °C (Method C).

Method A) and B):
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 2A) to cover specimen. Always store Pepsin at 2-8 °C.

Method A) Pepsin, RTU - Incubation at room temperature (20-25 °C):
Incubate for 5-15 minutes at room temperature (20-25 °C). An incubation time of 5-15 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. Continue to dehydration.
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.

Method B) Pepsin, RTU - Incubation at 37 °C:
Place specimen with Pepsin on a heating block at 37 °C – e.g. Dako Hybridizer – and incubate for 3-5 minutes. An incubation time of 3-5 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 diluted Wash Buffer for 3 minutes at room temperature (20-25 °C).
Replace Wash Buffer and soak sections for another 3 minutes. Continue to dehydration.
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.

Method C) Pepsin solution - Immersion of slides into 37 °C pepsin solution:
The kit contains reagents sufficient for four separate runs (60 mL pepsin solution, small container for six slides) or a single run (240 mL pepsin solution, large container for 24 slides).
Prepare the pepsin solution as described in section A.5.
Put lid on the container and equilibrate the pepsin solution to 37 (±2) °C in a water bath.
Ensure that the temperature has stabilized. Measure temperature inside the container with a calibrated thermometer to ensure correct temperature.
Tap off excess wash buffer. Immerse slides to the 37 (±2) °C pepsin solution and incubate for 20-30 minutes. An incubation time of 20-30 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 excess pepsin solution and soak sections in diluted Wash Buffer for 3 minutes at room temperature (20–25 °C).
Replace Wash Buffer and soak sections for another 3 minutes. Continue to dehydration.
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 IQISH Probe Mix**
HER2/CEN-17 IQISH Probe Mix separates into two phases while stored at -18 °C. Prior to use of Vial 3 ensure that only one phase is present by equilibrating the probe mix to room temperature (20-25 °C) followed by mixing. Thaw Vial 3 at room temperature (20-25 °C) for a maximum of 30 minutes (protect from strong light), then thoroughly whirl the vial for 15 seconds at 2500 rpm using a vortex mixer. Store Vial 3 at -18 °C immediately after use.

Apply 10 µL of HER2/CEN-17 IQISH 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.

**Remember to store Vial 3 at -18 °C immediately after use.**

The following step should be performed in a fume hood.

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.

Prepare Dako Hybridizer* (Code S2450) for a hybridization run. Make sure that Humidity Control Strips (Code S2452) are saturated and optimal for use. Start the Hybridizer and choose a program that will:

Denature at 66 °C for 10 minutes followed by hybridization at 45 °C for 60-120 minutes.

Place slides in the Hybridizer, make sure the lid is properly closed and start program. Please refer to Dako Hybridizer Instruction Manual for details.

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

Place slides on a flat metal or stone surface (heating block or on a block in a hybridization oven) preheated to 66 ± 1 °C. Denature for 10 minutes.

Place slides in a preheated humidified hybridization chamber. Cover the chamber with a lid and incubate at 45 °C for 60-120 minutes. Please note that a hybridization temperature of 37 °C is not suitable for use with the probes contained within this kit.

**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 one of the staining jars containing diluted Stringent Wash Buffer at room temperature in a fume hood and the other in a water bath. Heat water bath and the diluted Stringent Wash Buffer to 63 (±2) °C. Ensure that the temperature has stabilized. Cover jar with lid in order to stabilize the temperature and avoid evaporation. Measure temperature inside the water bath jar with a calibrated thermometer to ensure correct temperature. The Stringent Wash Buffer contains detergent and may become turbid at 63 °C; this will not affect performance.

Using forceps or gloves, take slides from the hybridization chamber and gently remove Coverslip Sealant as well as coverslip and place slides in the room temperature pre-wash jar, one at a time.

As soon as all coverslips have been removed, transfer slides from the room temperature, pre-wash jar to the 63 (±2) °C jar in the water bath.

Immediately after transferring the slides into the 63 (±2) °C jar in the water bath the count down should be started. Perform stringent wash for exactly 10 minutes.

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 -18-8 °C.
Quality Control - Breast

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 color.
   - 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 - Breast

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 tumor within the context of the H&E stained slide and evaluate the same area on the FISH stained slide. Scan several areas of tumor 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, 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 color. Score only those nuclei with one or more FISH signals of each color.
Signal counting guide

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Do not count. Nuclei are overlapping, not all areas of nuclei are visible</td>
</tr>
<tr>
<td>2</td>
<td>Two green signals, do not score nuclei with signals of only one color</td>
</tr>
<tr>
<td>3</td>
<td>Count as 3 green and 12 red signals (cluster estimation)</td>
</tr>
<tr>
<td>4</td>
<td>Count as 1 green and 1 red signal. Two signals of the same size and separate by a distance equal to or less than the diameter of one signal are counted as one</td>
</tr>
<tr>
<td>5</td>
<td>Do not count (over- or underdigested nuclei). Missing signals in the centre of nuclei (donut-shaped nuclei).</td>
</tr>
<tr>
<td>6</td>
<td>Count as 2 green and 3 red signals. Two signals of the same size and separate by a distance equal to or less than the diameter of one signal are counted as one</td>
</tr>
<tr>
<td>7</td>
<td>Count as 1 green and 5 red signals</td>
</tr>
<tr>
<td>8</td>
<td>Count as 3 green (1 green out of focus) and 3 red signals</td>
</tr>
<tr>
<td>9</td>
<td>Cluster of red signals hiding green signals, check the green signals with a specific FITC filter, or do not count</td>
</tr>
</tbody>
</table>

Record counts in a table as shown in Appendix 2.

Count 20 nuclei per tissue specimen, when possible from distinct tumor areas (28).

Calculate the \( \text{HER2}/\text{CEN-17} \) ratio by dividing the total number of red \( \text{HER2} \) signals by the total number of green \( \text{CEN-17} \) signals.

Specimens with a \( \text{HER2}/\text{CEN-17} \) ratio above or equal to 2 should be considered \( \text{HER2} \) gene amplified (3, 28-30).

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 - Breast**

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.

5. The performance of the HER2 IQFISH pharmDx assay has not been evaluated on breast tumor samples with microcalcifications.

**Performance Characteristics - Breast**

**Hybridization efficiency**

Hybridization efficiency of HER2 IQFISH pharmDx was investigated as part of the reproducibility study. From the total 180 formalin-fixed, paraffin-embedded tissue sections tested at the three study sites all 180 could be enumerated in accordance with product guidelines. Thus, the hybridization efficiency was 100%.

**Analytical sensitivity**

The sensitivity of the HER2/CEN-17 IQISH Probe Mix was investigated using 18 specimens of normal human breast epithelium. The ratio between the number of HER2 signals and CEN-17 signals was calculated based on a counting of 20 nuclei per specimen. HER2/CEN-17 ratio for the 18 specimens of normal human breast epithelium was between 0.97-1.08.

**Analytical specificity**

The HER2 DNA probes in the HER2/CEN-17 IQISH 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 IQISH 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 Dako QC procedures. A total of 275 metaphase spreads with distinct signals were evaluated for specific hybridization of the HER2 DNA and CEN-17 PNA probe mixes. In all 275 cases, the hybridization was specific for chromosome 17. No cross-hybridization to loci on other chromosomes was observed in any of the 275 cases. Probe specificity was thus 100% (275 of 275). To measure the assay’s ability to solely identify the target substances HER2 and CEN-17 without interference from other substances, studies were performed on tissue specimens of normal human breast epithelium using Vial 3 containing the hybridization buffer but without the probe mix. A total of 18 specimens were evaluated for presence of signals not related to the probe mix. No detection of other chromosome targets or interference with closely related substances was observed in any of the 18 specimens.
Robustness studies
The robustness of the HER2 IQFISH pharmDx assay was tested by varying pre-treatment time, temperature, and methods for heating of pre-treatment buffer (microwave oven or water bath), pepsin incubation time and method (RTU pepsin or immersion), denaturation temperature and time, hybridization time, and stringent wash time and temperature.

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

- Pretreatment Method A) Water bath for 10 minutes combined with each of the temperatures 95 °C, 95-99 °C, and 99 °C together with 9, 10 and 11 minutes at 95-99 °C
- Pretreatment Method B) Microwave oven for 9, 10, and 11 minutes at > 95 °C.
- Pepsin digestion Method A) with incubation times of 5, 10, and 15 minutes at room temperature (20-25 °C).
- Pepsin digestion Method B) with incubation times of 3, 4, and 5 minutes at 37 °C.
- Pepsin digestion Method C) with incubation times of 20, 25, and 30 minutes combined with each of the temperatures 35, 37, and 39 °C.
- Denaturation for 10 minutes combined with each of the temperatures 65, 66, and 67 °C together with 9, 10, and 11 minutes at 66 °C.
- Hybridization time of 60, 90, and 120 minutes at 45 °C.
- Stringency wash for 10 minutes combined with each of the temperatures 61, 63, and 65 °C together with 9, 10, and 11 minutes at 63 °C.

Note: For the robustness tests only one parameter in the staining procedure was changed at a time while all other parameters were kept constant. It is recommended to adhere to the time and temperatures indicated in the staining procedure.

Hybridization for 60 minutes gave high signal intensity though slightly reduced compared to 90 and 120 minutes hybridization. No significant difference in results was observed at the other time/temperature combinations.

The staining procedure for HER2 IQFISH pharmDx offers protocol variables for heat pre-treatment, pepsin digestion and hybridization time. Each unique combinatorial option has been validated with regard to HER2 status. Validation was performed on 10 FFPE human breast carcinoma specimens for each of the 12 possible combinatorial options. HER2 FISH pharmDx Kit (K5331) was used as reference. HER2/CEN-17 ratio for each individual specimen is shown in Figure 1. Cross tabulations between the 12 tests and the reference staining showed overall agreement of 100% (10/10) with lower and upper two-tailed 95% confidence limits at 78.3% and 100%, respectively. Kappa value was 1.00 and McNemars test showed absence of bias (two-tailed p-value of 1.00).
Breast Cancer

Figure 1. Individual HER2/CEN-17 ratios for each human breast carcinoma specimen obtained by staining performed with the 12 combinatorial staining protocol variations possible with the HER2 IQFISH pharmDx (K5731) (Test 1-12) and the HER2 FISH pharmDx Kit (K5331) reference (R). The horizontal line illustrates the cut-off value of 2.0.

Repeatability
The repeatability of the HER2/CEN-17 ratio was investigated with the HER2 IQFISH pharmDx assay using consecutive sections of nine human breast carcinoma specimens with either non-amplified or amplified HER2 gene status. Triplicate sections of each specimen were tested in the same run. The average coefficient of variation was 5.2% for non-amplified specimens (range from 1% to 8%) and 14% for amplified specimens (range from 7% to 20%).

A total of five consecutive sections of four human breast carcinoma specimens with different thickness (3, 4, 5, 6, and 7 µm) were tested with HER2 IQFISH pharmDx. The average coefficient of variation of the HER2/CEN-17 ratio was 7% (range from 6% to 9%).

Reproducibility
The day-to-day, observer-to-observer and site-to-site reproducibility were tested using HER2 IQFISH pharmDx. The study was designed as a three-site, blinded, randomized, comparative study of HER2/CEN-17 ratios using sections from 12 different formalin-fixed, paraffin-embedded breast cancer specimens with different levels of HER2 gene amplification. The 12 specimens had been tested with HercepTest™ and represented three specimens with HER2 IHC 0/1+, three specimens with HER2 IHC 2+, three specimens with HER2 IHC 3+, and three specimens with pre-determined HER2/CEN-17 FISH ratio between 1.5 and 2.5 obtained using HER2 IQFISH pharmDx. The specimens were stained and analyzed at three different study sites in USA and Europe. Each specimen was stained five times on five non-consecutive days and counted by two observers at each of the three study sites. A total of 180 stainings and 360 observations were made for the entire study. Variations in ratios between the different sites, days, and observers are illustrated in Figure 2. Data were analyzed using Box-Cox transformation. Estimated coefficient of variation for the non-amplified (210 observations) and amplified (150 observations) group of specimens was calculated to 0.095 and 0.089, respectively. Estimated coefficient of variation of the IHC 2+ category (90 observations) of specimens was calculated to 0.103, whereas the estimated coefficient of variation for the entire study was calculated to 0.130.
Breast Cancer

Figure 2. HER2/CEN-17 ratios obtained in the reproducibility study on HER2 IQFISH pharmDx including site-to-site, observer-to-observer, and day-to-day reproducibility.

Furthermore, the HER2 IQFISH pharmDx assay was tested for lot-to-lot reproducibility using three different lots of HER2 IQFISH pharmDx. Reproducibility was tested on nine different human breast carcinoma specimens with either non-amplified or amplified HER2 gene status. The average coefficient of variation for lot-to-lot reproducibility was 5% for non-amplified specimens (range from 2% to 8%) and 7.8% for amplified specimens (range from 5% to 11%).

Comparison study

Comparison of HER2 IQFISH pharmDx results with HER2 FISH pharmDx Kit results

HER2 IQFISH pharmDx (Code K5731) has been compared with HER2 FISH pharmDx Kit (Code K5331) in a comparative study on 121 human breast carcinoma tissue specimens. The specimens consisted of 61 HER2 non-amplified and 60 HER2 amplified cases with known HercepTest™ score as shown in Table 1.

Table 1. Distribution of specimens based on HercepTest™ score and HER2 FISH score obtained with HER2 FISH pharmDx Kit (K5331).

<table>
<thead>
<tr>
<th>HercepTest™ staining score</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>12</td>
<td>48</td>
<td>47</td>
<td>121</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HER2 FISH status</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplified</td>
<td>0</td>
<td>1</td>
<td>15</td>
<td>44</td>
<td>60</td>
</tr>
<tr>
<td>Non-amplified</td>
<td>14</td>
<td>11</td>
<td>33</td>
<td>3</td>
<td>61</td>
</tr>
<tr>
<td>Total FISH tested samples</td>
<td>14</td>
<td>12</td>
<td>48</td>
<td>47</td>
<td>121</td>
</tr>
</tbody>
</table>

Results of the cross tabulation of HER2 status obtained by the two assays with calculation of overall percent agreement (OPA), positive percent agreement (PPA), and negative percent agreement (NPA) with 95% confidence intervals are shown in Table 2. Correlation between the two assays is shown in Figure 3. The Kappa value was 0.98 with lower and upper limits for the 95% confidence interval at 0.95 and 1.00. The p-value for McNemars test was 1.00 indicating absence of bias between the two assays.
Table 2. Cross tabulation of HER2 gene status obtained using HER2 IQFISH pharmDx (K5731) and HER2 FISH pharmDx Kit (K5331).

<table>
<thead>
<tr>
<th>HER2 gene status (K5331)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-amplified</td>
<td>Amplified</td>
</tr>
<tr>
<td>Non-amplified</td>
<td>60</td>
</tr>
<tr>
<td>Amplified</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
</tr>
</tbody>
</table>

OPA: 99.2% [96.2%; 99.9%]
PPA: 100% [95.9%; 100%]
NPA: 98.4% [92.6%; 99.8%]

Figure 3. Correlation between HER2 FISH pharmDx Kit (K5331) and HER2 IQFISH pharmDx (K5731) on 121 breast cancer specimens.

One discrepant case was found as shown in Table 3. Re-examination of the slides revealed a small amplified area in the tissue section stained with K5731. This amplified area could not be found in the tissue section stained with K5331. The disagreement is therefore likely to be due to a heterogeneous tissue.
Table 3. Specimen with disagreement in HER2 gene status obtained by use of HER2 FISH pharmDx Kit (K5331) and HER2 IQFISH pharmDx (K5731).

<table>
<thead>
<tr>
<th>ID #</th>
<th>HercepTest™ score</th>
<th>HER2/CEN-17 ratio (K5331)</th>
<th>HER2/CEN-17 ratio (K5731)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>3+</td>
<td>1.43</td>
<td>2.21</td>
</tr>
</tbody>
</table>

Cross tabulation of HER2 status was also performed for the IHC 2+ subset of the breast cancer specimens (n = 48), as shown in Table 4. The Kappa value was 1 and McNemars test showed no bias between the two assays (p-value of 1.00).

Table 4. Cross tabulation of HER2 gene status obtained using HER2 IQFISH pharmDx (K5731) and HER2 FISH pharmDx Kit (K5331) for the IHC 2+ subset of specimens.

<table>
<thead>
<tr>
<th>HER2 gene status (K5331)</th>
<th>Non-amplified</th>
<th>Amplified</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-amplified</td>
<td>33</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>Amplified</td>
<td>0</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>15</td>
<td>48</td>
</tr>
</tbody>
</table>

OPA: 100% [94.9%; 100%]
PPA: 100% [84.8%; 100%]
NPA: 100% [92.7%; 100%]

Clinical utility in selection of patients for Herceptin® (trastuzumab) treatment

HER2 FISH pharmDx Kit was investigated in comparative studies with both the PathVision HER-2 DNA Probe Kit and Dako HercepTest™. Results of HER2 FISH testing are available for a total of 940 breast cancer specimens.

Comparison of HER2 FISH pharmDx Kit results with PathVysion HER-2 DNA Probe Kit test results

Three studies have been performed that compare the results of the HER2 FISH pharmDx Kit test to the results of the PathVysion HER-2 DNA Probe Kit test. 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.

Table 5. Summary data of FISH method comparison studies.

<table>
<thead>
<tr>
<th>Study designation</th>
<th>Concordance study (Danish specimens, N=190)</th>
<th>Concordance study (Japanese specimens, N=52)</th>
<th>Concordance study (French specimens, N=86) (31)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concordance (95% confidence interval)</td>
<td>93.68% (90.22% - 97.14%)</td>
<td>96.15%</td>
<td></td>
</tr>
<tr>
<td>Positive percent agreement (95% confidence interval)</td>
<td>86% (77.34% - 95.08%)</td>
<td>97%</td>
<td></td>
</tr>
<tr>
<td>Negative percent agreement (95% confidence interval)</td>
<td>97% (94.05% - 99.89%)</td>
<td>96%</td>
<td></td>
</tr>
</tbody>
</table>

* Comparison data were provided in the article, but the HER2 FISH assay was not identified.

There were a total of 12 discrepant test results between HER2 FISH pharmDx Kit test and PathVysion™ HER-2 DNA Probe test for the Danish clinical specimens.
Table 6 Summary of data for the 12 discrepant test results.

<table>
<thead>
<tr>
<th>ID#</th>
<th>HER2 FISH ratio</th>
<th>PathVysion ratio</th>
<th>HercepTest score</th>
<th>ID#</th>
<th>HER2 FISH ratio</th>
<th>PathVysion ratio</th>
<th>HercepTest score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>160</td>
<td>2.10*</td>
<td>1.51</td>
<td>2+</td>
<td>234</td>
<td>1.68</td>
<td>2.02*</td>
<td>2+</td>
</tr>
<tr>
<td></td>
<td>(1.82-2.51)</td>
<td>(1.39-1.68)</td>
<td></td>
<td></td>
<td>(1.38-1.83)</td>
<td>(1.84-2.29)</td>
<td></td>
</tr>
<tr>
<td>208</td>
<td>3.61</td>
<td>1.62</td>
<td>2+</td>
<td>284</td>
<td>1.44</td>
<td>2.21*</td>
<td>2+</td>
</tr>
<tr>
<td></td>
<td>(2.95-4.73)</td>
<td>(1.51-1.82)</td>
<td></td>
<td></td>
<td>(1.07-1.83)</td>
<td>(1.94-2.64)</td>
<td></td>
</tr>
<tr>
<td>306</td>
<td>2.20*</td>
<td>1.33</td>
<td>1+</td>
<td>423</td>
<td>1.7</td>
<td>2.15</td>
<td>1+</td>
</tr>
<tr>
<td></td>
<td>(1.79-2.24)</td>
<td>(1.18-1.44)</td>
<td></td>
<td></td>
<td>(1.52-1.95)</td>
<td>(2.02-2.45)</td>
<td></td>
</tr>
<tr>
<td>846</td>
<td>2.58</td>
<td>1.51</td>
<td>2+</td>
<td>474</td>
<td>1.44</td>
<td>2.55</td>
<td>2+</td>
</tr>
<tr>
<td></td>
<td>(2.06-3.50)</td>
<td>(1.42-1.76)</td>
<td></td>
<td></td>
<td>(1.16-1.83)</td>
<td>(2.38-3.26)</td>
<td></td>
</tr>
<tr>
<td>735</td>
<td>1.68</td>
<td></td>
<td></td>
<td>746</td>
<td>1.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(1.40-1.99)</td>
<td></td>
<td></td>
<td></td>
<td>(0.96-1.18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>837</td>
<td>1.52</td>
<td></td>
<td></td>
<td>881</td>
<td>1.83*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(1.48-1.79)</td>
<td></td>
<td></td>
<td></td>
<td>(1.15-2.69)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* CI of mean log ratios included 2.0. Numbers in parentheses are the 95% CI

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 HER-2 DNA Probe ratio. For the 4 instances where HER2 FISH pharmDx Kit was positive and PathVysion HER-2 DNA Probe was negative, no interval included the critical value of 2. Of the 8 instances where HER2 FISH pharmDx Kit was negative and PathVysion HER-2 DNA Probe 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 pharmDx™ Kit ratio. For the 4 instances where HER2 FISH pharmDx Kit was positive and PathVysion HER-2 DNA Probe was negative, 2 included the critical value of 2 (#160 and 306). Of the eight instances where HER2 FISH pharmDx Kit was negative and PathVysion HER-2 DNA Probe was positive, the 95% CI of 1 (#881) included the critical value of 2.

Comparison of HER2 FISH pharmDx Kit results with HercepTest™ results

Four studies comparing HER2 FISH pharmDx Kit to HercepTest™ results have been conducted. A total of 940 specimens have been compared, using 3+ staining score result as a positive IHC result in the HercepTest™ assay.

Table 7 Summary of HER2 FISH pharmDx Kit and IHC (HercepTest™) comparison studies.

<table>
<thead>
<tr>
<th>Study designation</th>
<th>Danish clinical specimens (N=682)</th>
<th>Japanese specimens (N=52)</th>
<th>French study (N=86) (31)</th>
<th>Danish pilot study (N=120) (32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concordance (95% confidence interval)</td>
<td>93.11% (91.21% - 95.01%)</td>
<td>96.15%</td>
<td>87.21%</td>
<td>93.33%</td>
</tr>
<tr>
<td>Positive percent agreement (95% confidence interval)</td>
<td>91% (87.39% - 94.57%)</td>
<td>96%</td>
<td>87%</td>
<td>84%</td>
</tr>
<tr>
<td>Negative percent agreement (95% confidence interval)</td>
<td>94% (92.12% - 96.46%)</td>
<td>96%</td>
<td>87%</td>
<td>97%</td>
</tr>
</tbody>
</table>
Distribution data of HercepTest™ and HER2 FISH test results for the Danish clinical specimens are presented in Table 8.
Table 8. Distribution of HER2 status by HercepTest™ and HER2 FISH pharmDx Kit.

<table>
<thead>
<tr>
<th>HercepTest™ staining score</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>221</td>
<td>267</td>
<td>84</td>
<td>248</td>
<td>820</td>
</tr>
<tr>
<td>%</td>
<td>27</td>
<td>33</td>
<td>10</td>
<td>30</td>
<td>100%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HER2 FISH status</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplified</td>
<td>0</td>
<td>8</td>
<td>17</td>
<td>222</td>
<td>247</td>
</tr>
<tr>
<td>Non-amplified</td>
<td>106</td>
<td>245</td>
<td>62</td>
<td>22</td>
<td>435</td>
</tr>
<tr>
<td>Total FISH tested samples</td>
<td>106</td>
<td>253</td>
<td>79</td>
<td>244</td>
<td>682</td>
</tr>
</tbody>
</table>

Clinical utility in selection of patients for PERJETA™ (pertuzumab) treatment

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

In the randomized study a total of 808 patients were enrolled, of whom 406 patients were randomized to the placebo treated group and 402 patients to the PERJETA™ treated group. The primary efficacy endpoint in the study was progression-free survival (PFS) based on tumor assessments by an independent review facility (IRF). At the time of the primary analysis of PFS, a total of 242 patients (59.6%) in the placebo treated group and 191 patients (47.5%) in the PERJETA™ treated group had IRF confirmed progressive disease or had died within 18 weeks of their last tumor assessment. The randomized study demonstrated a statistically significant improvement in IRF-assessed PFS (hazard ratio [HR] = 0.62, 95% CI = 0.51; 0.75, p < 0.0001) in the PERJETA™ treated group compared with the placebo treated group, and an increase in median PFS of 6.1 months (median PFS of 12.4 months in the placebo treated group vs. 18.5 months in the PERJETA™ treated group). Figure 4 shows the Kaplan-Meier Curve of IRF-assessed PFS.
Breast Cancer

Figure 4. Kaplan-Meier Curve of IRF-assessed Progression Free Survival.

Clinical utility in selection of patients for KADCYLA™ (ado-trastuzumab emtansine) treatment
The clinical safety and efficacy of KADCYLA™ (ado-trastuzumab emtansine) has been demonstrated in a phase III study (EMILIA) (22). The study was designed as a randomized, multicenter, international, two-arm, open-label clinical trial in patients with centrally confirmed HER2-positive, unresectable locally advanced or metastatic breast cancer who have previously received a taxane and trastuzumab and have progressive disease at study entry. After inclusion in the study the patients were randomized 1:1 to receive lapatinib and capecitabine or ado-trastuzumab emtansine. In the study, HER2 positivity was defined as being IHC positive (3+) and/or FISH positive. The majority of patients (881/991) had a test result available with both Dako HercepTest™ and Dako HER2 FISH pharmDx Kit. Cases that were IHC negative (0, 1+) that were FISH positive (HER2/CEN-17 ≥ 2.0) were enrolled. However limited data were available for cases that were positive by FISH but negative by IHC (11/991) (no direct evidence of protein overexpression) and as such, caution should be exercised when interpreting these results.

In the phase III study a total of 991 patients were enrolled, of whom 496 patients were randomized to the lapatinib and capecitabine group and 495 patients to the ado-trastuzumab emtansine group. The co-primary efficacy endpoints in the study were progression-free survival (PFS) based on tumor assessments by an independent review committee (IRC) and overall survival (OS). As of the clinical data cutoff of 14 January 2012, 182 patients (37.1%) in the ado-trastuzumab emtansine arm and 125 patients (25.6%) in the lapatinib plus capecitabine arm were still alive and on treatment. The randomized phase III study showed that treatment with ado-trastuzumab emtansine was associated with a 35% reduction in the risk of disease progression or death (hazard ratio of (HR) = 0.650 (95% CI = 0.549, 0.771; p < 0.0001)) compared to lapatinib and capecitabine. The Kaplan-Meier curve showed a clear separation at approximately 4 months that was maintained from that point onward (Figure 5). A 50% relative increase in median PFS (by IRC) was observed in patients treated with ado-trastuzumab...
emtansine compared with patients treated with lapatinib plus capecitabine (9.6 months vs. 6.4 months).

**Figure 5.** Kaplan-Meier Curve of IRC-assessed Progression Free Survival.

At the time of PFS analysis, 223 patients had died. More deaths occurred in the lapatinib plus capecitabine arm (26%) compared with the KADCYLA arm (19%), however the results of this interim OS analysis did not meet the pre-specified stopping boundary for statistical significance. A second interim OS analysis was planned for when at least 50% of the target 632 events for final analysis had occurred. At the time of the second interim analysis, 331 events (representing 52.4% of targeted events) had occurred. The co-primary endpoint of OS was met; OS was significantly improved in patients receiving KADCYLA (HR = 0.682, 95% CI: 0.548, 0.849, p = 0.0006; See Figure 6). This result crossed the pre-specified efficacy stopping boundary (HR = 0.727 or p = 0.0037) and represents the confirmatory analysis for OS. The median duration of survival was 30.9 months in the KADCYLA arm vs. 25.1 months in the lapatinib plus capecitabine arm.
Figure 6. Kaplan-Meier Curve of Overall Survival

Kaplan-Meier Plot of Overall Survival
Randomized Subjects

Median Time (mo)
Hazard Ratio
(95% CI)
Logrank p-value

Lap+Cap
T-DM1
25.1
33.9
0.682
0.560
0.849
0.0006

Number at Risk:
Lap+Cap
T-DM1
496
485
471
485
453
474
435
457
403
439
368
349
297
293
240
242
159
157
133
136
119
111
86
80
63
62
45
36
27
28
17
13
7
4

Duration of Survival (months)

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

<table>
<thead>
<tr>
<th>Problem</th>
<th>Probable Cause</th>
<th>Suggested Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. No signals or weak signals</td>
<td>1a. Kit has been exposed to high temperatures during transport or storage</td>
<td>1a. Check storage conditions. Ensure that dry ice was present when the consignment was received. Ensure that vial 3 has been stored at -18 °C in the dark. Ensure that vials 2A and 5 have been stored at maximum 2-8 °C, and that vials have been stored in the dark.</td>
</tr>
</tbody>
</table>
|                                              | 1b. Microscope not functioning properly  
  - Inappropriate filter set  
  - Improper lamp  
  - Mercury lamp too old  
  - Dirty and/or cracked collector lenses  
  - Unsuitable immersion oil  
  1c. Faded signals  
  1d. Pre-treatment conditions incorrect  
  1e. Evaporation of Probe Mix during hybridization                                                                 | 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. Avoid long microscopic examination and minimize exposure to strong light sources.  
  1d. Ensure that the recommended pre-treatment temperature and time are used.  
  1e. Ensure sufficient humidity in the hybridization chamber. |
<p>| 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. |</p>
<table>
<thead>
<tr>
<th>Problem</th>
<th>Probable Cause</th>
<th>Suggested Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>4b. Air bubbles caught during Probe Mix application or mounting</td>
<td>4b. Avoid air bubbles. If observed, gently tap them away using forceps.</td>
<td></td>
</tr>
<tr>
<td>5. Excessive background staining</td>
<td>5a. Inappropriate tissue fixation</td>
<td>5a. Ensure that only formalin-fixed, paraffin-embedded tissue sections are used.</td>
</tr>
<tr>
<td></td>
<td>5b. Paraffin incompletely removed</td>
<td>5b. Follow the deparaffinization and rehydration procedures outlined in Section B.2.</td>
</tr>
<tr>
<td></td>
<td>5c. Stringent wash temperature too low</td>
<td>5c. Ensure that the stringent wash temperature is 63 (±2) °C.</td>
</tr>
<tr>
<td></td>
<td>5d. Prolonged exposure of hybridized section to strong light</td>
<td>5d. Avoid long microscopic examination and minimize exposure to strong light.</td>
</tr>
<tr>
<td>6. Poor tissue morphology</td>
<td>6a. Incorrect Pepsin treatment</td>
<td>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.</td>
</tr>
<tr>
<td></td>
<td>6b. Incorrect pre-treatment conditions may result in unclear or cloudy appearance</td>
<td>6b. Ensure that the recommended pre-treatment temperature and time are used.</td>
</tr>
<tr>
<td></td>
<td>6c. Too long Pepsin treatment or very thin section thickness may cause ghost cells or donut cells to appear</td>
<td>6c. Shorten the Pepsin incubation time. See section B.3, step 2. Ensure that the section thickness is 4-6 µm.</td>
</tr>
<tr>
<td>7. High level of green auto fluorescence on slide including areas without FFPE tissue</td>
<td>7. Use of old glass slides</td>
<td>7. Ensure that the coated glass slides (Dako Silanized Slides, Code S3003, or poly-L-lysine-coated slides) have not passed expiry date.</td>
</tr>
</tbody>
</table>

**NOTE:** If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please call Dako Technical Services for further assistance.
# Appendix 1 - Breast

**HER2 IQFISH pharmDx, Code K5731**

## Protocol Checklist

<table>
<thead>
<tr>
<th>Staining Run Log ID: ________________</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of the run: ____________________</td>
</tr>
<tr>
<td><strong>HER2 IQFISH pharmDx, K5731 Lot:</strong></td>
</tr>
<tr>
<td>Specimen ID: ________________________</td>
</tr>
<tr>
<td>Equipment ID: ________________________</td>
</tr>
<tr>
<td>Date of dilution/expiration of the 1x Wash Buffer (Vial 6 diluted 1:20): __________ / __________</td>
</tr>
<tr>
<td>Tissue fixed in neutral buffered formalin</td>
</tr>
</tbody>
</table>

## Step 1: Pre-Treatment

<table>
<thead>
<tr>
<th>Date of dilution/expiration of the Pre-Treatment Solution (Vial 1 diluted 1:20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured temperature of Pre-Treatment Solution (95-99 °C) if water bath is used for heating</td>
</tr>
<tr>
<td>Pre-treatment (10 minutes), and cooling (15 minutes)</td>
</tr>
<tr>
<td>Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes)</td>
</tr>
</tbody>
</table>

## Step 2: Pepsin

<table>
<thead>
<tr>
<th>Duration of Pepsin (Vial 2) treatment at 37 °C or</th>
<th>Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of Pepsin (Vial 2) treatment at room temperature or</td>
<td>Minutes</td>
</tr>
<tr>
<td>Duration of Pepsin immersion at 37 (±2) °C</td>
<td>Minutes</td>
</tr>
<tr>
<td>Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes)</td>
<td></td>
</tr>
<tr>
<td>Dehydrate slides (3 x 2 minutes) in graded series of ethanol and air dry</td>
<td></td>
</tr>
</tbody>
</table>

## Step 3: HER2/CEN-17 IQISH Probe Mix

| Apply Probe Mix (Vial 3), coverslip and seal with Coverslip Sealant |
| Measured denaturation temperature (66 ±1 °C) | °C |
| Denaturation for 10 minutes |
| Measured hybridization temperature (45 °C) | °C |
| Hybridization time (60 to 120 minutes) | Minutes |

## Step 4: Stringent Wash

| Date of dilution/expiration of the Stringent Wash Buffer (Vial 4 diluted 1:20) |
| Measured temperature of Stringent Wash Buffer (63 ±2 °C) | °C |
| Stringent wash (10 minutes) after removing the coverslips |
| Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes) | °C |
| Dehydrate slides (3 x 2 minutes) in graded series of ethanol and air dry | Minutes |

## Step 5: Mounting

| Apply 15 μL of Fluorescence Mounting Medium (Vial 5) and coverslip |

### Comments:

________________________________________

Date and signature, Technician: ____________________________
Appendix 2 - Breast

HER2 IQFISH pharmDx, Code K5731

Scoring Scheme

Staining Run Log ID: __________

Date of the run: __________________

HER2 IQFISH pharmDx K5731  Lot: __________  Specimen ID: _________________

<table>
<thead>
<tr>
<th>Nucleus No.</th>
<th>HER2 score (red)</th>
<th>CEN-17 score (green)</th>
<th>Nucleus No.</th>
<th>HER2 score (red)</th>
<th>CEN-17 score (green)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td></td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>12</td>
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<td>12</td>
<td>12</td>
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<tr>
<td>3</td>
<td>13</td>
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<td>4</td>
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</tr>
<tr>
<td>10</td>
<td>20</td>
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<td>20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Total (1-10)</td>
<td></td>
<td></td>
<td>Total (11-20)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th></th>
<th>HER2</th>
<th>CEN-17</th>
<th>HER2/CEN-17 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total score (1-20)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- 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.
Appendix 3 - Breast

HER2 IQFISH pharmDx, Code K5731
Fluorescence Microscope Specifications
Dako recommends the following equipment for use with HER2 IQFISH pharmDx, K5731:

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. Dako recommends the use of a specific DAPI filter in combination with a high quality Texas Red/FITC double filter.
   - DAPI filter.
   - Texas Red/FITC double filter.
   - Texas Red and FITC single filters can be used for confirmation.

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Excitation Wavelength</th>
<th>Emission Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC</td>
<td>495 nm</td>
<td>520 nm</td>
</tr>
<tr>
<td>Texas Red</td>
<td>596 nm</td>
<td>615 nm</td>
</tr>
</tbody>
</table>

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 Dako 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.
Summary and Explanation - Gastric

The human HER2 gene (also known as ERBB2 or NEU) is located on chromosome 17 and encodes the HER2 protein or p185HER2. The HER2 protein is a membrane receptor tyrosine kinase with homology to the epidermal growth factor receptor (EGFR or HER1) (1-2). The HER2 gene is present in 2 copies in all normal diploid cells.

Overexpression of the HER2 protein and amplification of the HER2 gene in gastric cancer have been shown in a large number of studies (reviewed in (33)). HER2 positivity can be detected in approximately 20 % of the patients by either IHC or FISH (33). Preclinical in vitro and in vivo studies have demonstrated that trastuzumab (Herceptin®) is effective in different gastric cancer models, thus leading to the initiation of several clinical studies (33-37).

All of the patients in the phase III BO18255 (ToGA “An open-label, randomized, multicenter, phase III study of trastuzumab in combination with a fluoropyrimidine and cisplatin versus chemotherapy alone as first-line therapy in patients with HER2 positive advanced gastric cancer”) study sponsored by Hoffmann-La Roche AG were selected using Dako HercepTest™ (IHC) and Dako HER2 FISH pharmDx Kit (FISH) with HER2 positivity defined as IHC 3+ or FISH+ (HER2/CEN-17 ≥ 2.0). The study demonstrated the clinical utility of both HercepTest™ (IHC) and HER2 FISH pharmDx Kit (FISH) for the assessment of HER2 status in patients with advanced gastric or gastroesophageal junction adenocarcinoma for whom trastuzumab treatment is being considered (38).

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

Principle of Procedure - Gastric

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

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 or at 37 °C for 3-5 minutes or by immersing the slides into Pepsin solution at 37 °C for 20-30 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) (23) 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. 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), tumor 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 - Gastric

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 2A, 10 µL per slide of Vial 3, and 15 µL per slide of Vial 5). The solutions in Vial 3 and Vial 5 are viscous and may have to be centrifuged shortly in a microcentrifuge in order to be able to collect all of the provided reagent.

The kit provides materials sufficient for 10 individual staining runs (four separate runs, when using the pepsin immersing method).

*HER2 IQFISH pharmDx* 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 *HER2 IQFISH pharmDx*.

Vial 1

**PRE-TREATMENT SOLUTION (20x)**

**Pre-Treatment Solution (20x)**

150 mL, concentrated 20x

MES (2-[N-morpholino]ethanesulphonic acid) buffer.

Vial 2A

**PEPSIN**

**Pepsin**

4 x 6.0 mL, ready-to-use

Pepsin solution, pH 2.0; contains stabilizer and an antimicrobial agent.

Vial 2B

**PEPSIN DILUENT (10x)**

**Pepsin Diluent (10x)**

24 mL, concentrated 10x

Dilution buffer, pH 2.0; contains an antimicrobial agent.

Vial 3

**HER2/CEN-17 IQISH PROBE MIX**

**HER2/CEN-17 IQISH Probe Mix**

0.2 mL, ready-to-use

Mix of Texas Red-labelled *HER2* DNA probes and fluorescein-labelled CEN-17 PNA probes; supplied in IQISH hybridization buffer.

Vial 4

**STRINGENT WASH BUFFER (20x)**

**Stringent Wash Buffer (20x)**

150 mL, concentrated 20x

SSC (saline-sodium citrate) buffer with detergent (Tween-20)

Vial 5

**FLUORESCENCE MOUNTING MEDIUM**

**Fluorescence Mounting Medium**

0.4 mL, ready-to-use

Fluorescence mounting medium with 500 µg/L DAPI (4′,6-diamidine-2-phenylindole).

Vial 6

**WASH BUFFER (20x)**

**Wash Buffer (20x)**

500 mL, concentrated 20x

Tris/HCl buffer.
Coverslip Sealant
1 tube, ready-to-use
Solution for removable sealing of coverslips.

NOTE: Kit accessory reagents: Pre-Treatment Solution, Pepsin, Pepsin Diluent, Stringent Wash Buffer, Fluorescence Mounting Medium, Wash Buffer and Coverslip Sealant, can be substituted by identical Dako retail reagents in Dako Histology FISH Accessory Kit, Code K5799.

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
Dako Hybridizer (Code S2450)*
Heating block or hybridization oven for denaturation (66 (±1) °C)*
Humid hybridization chamber*
Microcentrifuge (tabletop centrifuge for spinning down probe and mounting media)
Slides, Dako Silanized Slides, Code S3003, or poly-L-lysine-coated slides (see Specimen Preparation)
Staining jars or baths
Timer (capable of 2-15 minute intervals)
Vortex mixer
Water bath with lid (capable of maintaining 37(±2) °C, 63 (±2) °C and from 95 °C to 99 °C)
Microwave oven with sensing capability if pre-treatment is performed using microwave oven (see Section B.3. Staining protocol. Step 1: Pre-treatment, Method B)

* Heating block or hybridization oven for denaturation (66 (±1) °C) and hybridization (45 °C) together with a humid hybridization chamber can be used as an alternative to Dako Hybridizer.

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 as light source should be used. Other light sources are not recommended with these filters.
Microscope slide folder (cardboard tray for 20 slides with hinged cover or similar)
Precautions - Gastric

1. For in vitro diagnostic use.
2. For professional users.
3. Vial 1, Pre-Treatment Solution (20x), does not require hazard labeling. Safety Data Sheet (SDS) is available for professional users on request.
4. Vial 2A, Pepsin, contains 5-10% propan-2-ol, 0.1-1% pepsin A, and <0.1% 3(2H)-Isothiazolone, 5-chloro-2-methyl-, mixt. with 2-methyl-3(2H)-isothiazolone. Vial 2A is labeled:

Danger

H314 Causes severe skin burns and eye damage.
H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled.
H317 May cause an allergic skin reaction.
H373 May cause damage to organs through prolonged or repeated exposure.
P280 Wear protective gloves. Wear eye or face protection. Wear protective clothing.
P285 In case of inadequate ventilation wear respiratory protection.
P260 Do not breathe vapor.
P264 Wash hands thoroughly after handling.
P272 Contaminated work clothing should not be allowed out of the workplace.
P314 Get medical attention if you feel unwell.
P304 + P340 + P310 IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing. Immediately call a POISON CENTER or physician.
P342 + P311 If experiencing respiratory symptoms: Call a POISON CENTER or physician.
P301 + P310 + P330 + P331 IF SWALLOWED: Immediately call a POISON CENTER or physician. Rinse mouth. Do NOT induce vomiting.
P303 + P361 + P353 + P363 + P310 IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water or shower. Wash contaminated clothing before reuse. Immediately call a POISON CENTER or physician.
P302 + P352 IF ON SKIN: Wash with plenty of soap and water.
P333 + P313 If skin irritation or rash occurs: Get medical attention.
P305 + P351 + P338 + P310 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER or physician.
P405 Store locked up.
P501 Dispose of contents and container in accordance with all local, regional, national and international regulations.
5. Vial 2B, Pepsin Diluent (19x), contains 30-60% propan-2-ol, and 5-10% 2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride. Vial 2B is labeled:

**Danger**

H325 Highly flammable liquid and vapor.
H319 Causes serious eye irritation.
H335 May cause respiratory irritation.
H336 May cause drowsiness and dizziness.
P373 May cause damage to organs through prolonged or repeated exposure.
P280 Wear protective gloves. Wear eye or face protection.
P210 Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.
P241 Use explosion-proof electrical, ventilating, lighting and all material-handling equipment.
P242 Use only non-sparking tools.
P243 Take precautionary measures against static discharge.
P233 Keep container tightly closed.
P271 Use only outdoors or in a well-ventilated area.
P260 Do not breathe vapor.
P264 Wash hands thoroughly after handling.
P314 Get medical attention if you feel unwell.
P304 + P340 + P312 IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing. Call a POISON CENTER or physician if you feel unwell.
P303 + P361 + P353 IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water or shower.
P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337 + P313 If eye irritation persists: Get medical attention.
P405 Store locked up.
P403 Store in a well-ventilated place.
P235 Keep cool.
P501 Dispose of contents and container in accordance with all local, regional, national and international regulations.

6. Vial 3, HER2/CEN-17 IQISH Probe Mix, contains 10-30% ethylene carbonate, and 1-5% sodium chloride. Vial 3 is labeled:

**Warning**

H319 Causes serious eye irritation.
P280 Wear eye or face protection.
P264 Wash hands thoroughly after handling.
P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337 + P313 If eye irritation persists: Get medical attention.
7. Vial 4, Stringent Wash Buffer (20x), contains 10-30% sodium chloride, and 10-30% 2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride. Vial 4 is labeled:

![Warning]

**Warning**

H319 Causes serious eye irritation.
H315 Causes skin irritation.
P280 Wear protective gloves. Wear eye or face protection.
P264 Wash hands thoroughly after handling.
P302 + P352 + P362-2 + P363 IF ON SKIN: Wash with plenty of soap and water. Take off contaminated clothing. Wash contaminated clothing before reuse.
P332 + P313 If skin irritation occurs: Get medical attention.
P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337 + P313 If eye irritation persists: Get medical attention.

8. Vial 6, Wash Buffer (20x), contains 10-30% sodium chloride, and 10-30% trometamol. Vial 6 is labeled:

![Warning]

**Warning**

H319 Causes serious eye irritation.
H315 Causes skin irritation.
P280 Wear protective gloves. Wear eye or face protection.
P264 Wash hands thoroughly after handling.
P302 + P352 + P362-2 + P363 IF ON SKIN: Wash with plenty of soap and water. Take off contaminated clothing. Wash contaminated clothing before reuse.
P332 + P313 If skin irritation occurs: Get medical attention.
P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337 + P313 If eye irritation persists: Get medical attention.

9. Coverslip Sealant contains 60-100% naphtha (petroleum), hydrotreated light, and is labeled:

![Danger]

**Danger**

H225 Highly flammable liquid and vapor.
H304 May be fatal if swallowed and enters airways.
P280 Wear protective gloves. Wear eye or face protection.
P210 Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.
P241 Use explosion-proof electrical, ventilating, lighting and all material-handling equipment.
P242 Use only non-sparking tools.
P243 Take precautionary measures against static discharge.
P233 Keep container tightly closed.
P301 + P310 + P331 IF SWALLOWED: Immediately call a POISON CENTER or physician. Do NOT induce vomiting.
P303 + P361 + P353 IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water or shower.
P405 Store locked up.
10. 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 (24). 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.

11. 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 (24). 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.

12. Minimize microbial contamination of reagents to avoid erroneous results.

13. Incubation times and temperatures, or methods other than those specified, may give erroneous results.

14. Tissue fixation methods and thickness of specimen other than those specified may affect tissue morphology and/or signal intensity.

15. Avoid evaporation of HER2/CEN-17 Probe Mix during hybridization by ensuring sufficient humidity in the hybridization chamber.

16. Reagents have been optimally diluted. Further dilution may result in loss of performance.

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

18. Due to the heterogeneous nature of gastric cancer specimens it is important to perform a thorough inspection of the entire specimen to evaluate signal distribution before selecting the area for signal enumeration.

19. It is not recommended to evaluate very small specimens, i.e. specimens must have intact morphology and sufficient nuclei for enumeration.

20. If HER2 FISH analysis is performed on a biopsy specimen, multiple (7-8) evaluable biopsies from different regions of the tumor should be analyzed to ensure reliable determination of HER2 status.

21. For identification of all tissue cores in a biopsy sample it is important to inspect the H&E stained slide.

22. HER2 gene amplification and HER2 protein overexpression are not as well correlated in gastric cancer as with breast cancer; therefore a single method should not be used to determine HER2 status.

23. Only clean staining jars should be used for the pepsin immersion method (Step 2, method C).

**Storage - Gastric**

Store the HER2/CEN-17 IQISH Probe Mix (Vial 3) at -18 °C in the dark. All other reagents can be stored at 2-8 °C in the dark. All reagents tolerate frozen storage. Freezing and thawing the probe mix for up to 10 times does not affect performance.

The Pepsin, HER2/CEN-17 IQISH Probe Mix, and Fluorescence Mounting Medium (Vials 2A, 3 and 5) may be affected adversely if exposed to heat. Do not leave these components at room temperature.

The HER2/CEN-17 IQISH 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 (25).
There are no obvious signs indicating 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 IQFISH pharmDx is suspected, contact Dako Technical Services immediately.

**Specimen Preparation - Gastric**

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

**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. Biopsy specimens were fixed for 6-8 hours in the ToGA trial (for study reference, refer to (38)). 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) (26-27). Other fixatives are not suitable.

Tissue specimens should be cut into sections of 3-6 µm.

The slides required for HER2 gene amplification analysis and verification of tumor presence should be prepared at the same time. A minimum of 2 serial sections is recommended, 1 section for tumor 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 Dako Silanized Slides, Code S3003, 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 - Gastric**

**A. Reagent Preparation - Gastric**

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 20x) 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 20x) 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 20x) 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.

**A.5 Pepsin Solution**
A pepsin solution is only needed when using pepsin immersing method (Method C).
Prepare pepsin solution as follows;
For a six slide capacity container prepare 60 mL pepsin solution:
Add 48 mL room temperature (20-25 °C) distilled or deionized water to the container.
Add 6 mL cold (2-8 °C) Pepsin Diluent (10x) (Vial 2B) to the container.
Add 6 mL cold (2-8 °C) Pepsin (Vial 2A) to the container.
Put lid on the container and equilibrate the pepsin solution to 37 (±2) °C in a water bath.

For a 24 slide capacity container prepare 240 mL pepsin solution:
Add 192 mL room temperature (20-25 °C) distilled or deionized water to the container.
Add 24 mL cold (2-8 °C) Pepsin Diluent (10x) (Vial 2B) to the container.
Add 24 mL cold (2-8 °C) Pepsin (Vial 2A) to the container.
Put lid on the container and equilibrate the pepsin solution to 37 (±2) °C in a water bath.

Equilibrated pepsin solution should be used within 5 hours.
B. Staining Procedure - Gastric

B.1 Procedural notes
The user should read these instructions carefully and become familiar with all components prior to use (see Precautions).

All reagents should be equilibrated to the relevant temperature prior to use as follows:

**Vial 1:** The diluted Pre-Treatment Solution should be equilibrated to 95-99 °C if water bath is used for pre-treatment (Section B.3. Staining protocol, Step 1: Pre-Treatment Method A). If microwave oven with sensing capability is used for pre-treatment (Section B.3. Staining protocol, Step 1: Pre-Treatment, Method B) the diluted Pre-Treatment Solution should be equilibrated to room temperature 20-25 °C.

**Vial 2A:** Pepsin should be applied at 2-8 °C (Section B.3, Staining protocol, Step 2 Method A and B) and kept cold continuously.

**Vial 2B:** Pepsin Diluent (10x) should be applied at 2-8 °C (Section B.3, Staining protocol, Step 2 Method C).

**Vial 3:** HER2/CEN-17 IQISH Probe Mix separates into two phases while stored at -18 °C. Prior to use of Vial 3 ensure that only one phase is present by equilibration to room temperature (20-25 °C) followed by mixing; Thaw Vial 3 by placing it at room temperature for maximum 30 minutes (protect from strong light), then thoroughly whirl the vial for 15 seconds at 2500 rpm using a vortex mixer. Store Vial 3 at -18 °C immediately after use.

**Vial 4:** The Diluted Stringent Wash Buffer; one jar should be equilibrated to room temperature, another jar should be equilibrated to 63 (±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 temperature 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

#### Step 1: Pre-Treatment

Pre-treatment can be performed either by using water bath as described in method A) or, alternatively, by use of microwave oven with sensing capability as described in method B).

**Method A) Pre-treatment using water bath:**

Fill staining jars, e.g. Coplin jars, with the diluted Pre-Treatment Solution (see INSTRUCTIONS FOR USE, Section A.1). Place staining jars containing diluted Pre-Treatment Solution in water bath. Heat water bath and the Pre-Treatment Solution to 95-99 ºC. 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 ºC.

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 ºC).

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.

**Method B) Pre-treatment using microwave oven with sensing capability:**

Fill a plastic jar with diluted room temperature (20-25 ºC) Pre-Treatment Solution. Immerse the deparaffinized sections in Pre-Treatment Solution, cover the jar with a punctured lid and place it in the microwave oven. Select the boiling sensor function and a program that runs for 10 minutes after boiling temperature has been reached*.

Following the 10 minutes incubation take the jar with slides out of the oven, remove the lid and cool for 15 minutes at room temperature. Transfer the slides to a jar with diluted Wash Buffer and soak for 3 minutes at room temperature (20-25 ºC). Replace Wash Buffer and soak sections for another 3 minutes.

*The use of a microwave oven with a sensing capability means that the oven must include a sensor and programs which initially heat the Pre-Treatment Solution to the boiling point and subsequently maintain the required pre-treatment temperature (above 95 ºC) while counting down the preset time (10 (±1) minutes). Some microwave oven models with sensing capability may not include the possibility to freely set a count-down time. If the model only includes pre-set programs, be sure to select a program which maintain the required pre-treatment temperature (above 95 ºC) for at least 10 (±1) minutes and manually stop the program after 10 (±1) minutes.

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

#### Step 2: Pepsin, ready-to-use (RTU) or pepsin solution

Pepsin incubation can be performed by direct application of RTU pepsin drops to the slides either at room temperature (20-25 ºC) (Method A) or at 37 ºC (Method B). Alternatively, slides can be immersed into a pepsin solution and incubated at 37 (±2) ºC (Method C).
**Gastric Cancer**

Method A) and B):
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 2A) to cover specimen. Always store Pepsin at 2-8 °C.

**Method A) Pepsin, RTU - Incubation at room temperature (20-25 °C):**
Incubate for 5-15 minutes at room temperature (20-25 °C). An incubation time of 5-15 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. Continue to dehydration. 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.

**Method B) Pepsin, RTU - Incubation at 37 °C:**
Place specimen with Pepsin on a heating block at 37 °C – e.g. Dako Hybridizer – and incubate for 3-5 minutes. An incubation time of 3-5 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 diluted Wash Buffer for 3 minutes at room temperature (20-25 °C).

Replace Wash Buffer and soak sections for another 3 minutes. Continue to dehydration. 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.

**Method C) Pepsin solution - Immersion of slides into 37 °C pepsin solution:**
The kit contains reagents sufficient for four separate runs (60 mL pepsin solution, small container for six slides) or a single run (240 mL pepsin solution, large container for 24 slides). Prepare the pepsin solution as described in section A.5.

Put lid on the container and equilibrate the pepsin solution to 37 (±2) °C in a water bath. Ensure that the temperature has stabilized. Measure temperature inside the container with a calibrated thermometer to ensure correct temperature.

Tap off excess wash buffer. Immerse slides into the 37 (±2) °C pepsin solution and incubate for 20-30 minutes. An incubation time of 20-30 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 excess pepsin solution and soak sections in diluted Wash Buffer for 3 minutes at room temperature (20–25 °C).

Replace Wash Buffer and soak sections for another 3 minutes. Continue to dehydration. 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 IQISH Probe Mix**

HER2/CEN-17 IQISH Probe Mix separates into two phases while stored at -18 °C. Prior to use of Vial 3 ensure that only one phase is present by equilibrating the probe to room temperature (20-25 °C) followed by mixing. Thaw Vial 3 at room temperature (20-25 °C) for a maximum 30 minutes (protect from strong light), then thoroughly whirl the vial for 15 seconds at 2500 rpm using a vortex mixer. Immediately store Vial 3 at -18 °C after use.
Apply 10 µL of HER2/CEN-17 IQISH 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.

**Remember to store Vial 3 at -18 °C immediately after use.**

The following step should be performed in a fume hood.

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.

Prepare Dako Hybridizer* (Code S2450) for a hybridization run. Make sure that Humidity Control Strips (Code S2452) are saturated and optimal for use. Start the Hybridizer and choose a program that will:

- Denature at 66 °C for 10 minutes followed by hybridization at 45 °C for 60-120 minutes. Place slides in the Hybridizer, make sure the lid is properly closed and start program. Please refer to Dako Hybridizer Instruction Manual for details.

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

- Place slides on a flat metal or stone surface (heating block or on a block in a hybridization oven) preheated to 66 (±1) °C. Denature for 10 minutes. Place slides in a preheated humidified hybridization chamber. Cover the chamber with a lid and incubate at 45 °C for 60-120 minutes. Please note that a hybridization temperature of 37 °C is not suitable for use with the probes contained within this kit.

**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 one of the staining jars containing diluted Stringent Wash Buffer at room temperature in a fume hood and the other in a water bath. Heat water bath and the diluted Stringent Wash Buffer to 63 (±2) °C. Ensure that the temperature has stabilized. Cover jar with lid in order to stabilize the temperature and avoid evaporation. Measure temperature inside the water bath jar with a calibrated thermometer to ensure correct temperature. The Stringent Wash Buffer contains detergent and may become turbid at 63 °C; this will not affect performance.

Using forceps or gloves, take slides from the hybridization chamber and gently remove Coverslip Sealant as well as coverslip and place slides in the room temperature pre-wash jar, one at a time.

As soon as all coverslips have been removed, transfer slides from the room temperature pre-wash jar to the 63 (±2) °C jar in the water bath.

Immediately after transferring the slides into the 63 (±2) °C jar in the water bath the count down should be started. Perform stringent wash for exactly 10 minutes at 63 (±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 -18-8 °C.

Quality Control - Gastric
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 color.
   - 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. The minimum number of assessable tumor cells is 20.
5. 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 - Gastric

Assessable tissue
Locate the tumor within the context of the H&E stained slide and evaluate the same area on the FISH stained slide (in the DAPI filter). Only specimens from patients with gastric or gastroesophageal junction adenocarcinoma should be analyzed. In cases with intestinal metaplasia and adenocarcinoma in the same specimen, only the carcinoma component should be scored. Avoid areas of heavy inflammation, necrosis and areas where the nuclear borders are ambiguous. Do not include nuclei that require subjective judgment. Do not include nuclei with weak signal intensity and non-specific or high background.

Begin with a microscope evaluation of the complete FISH stained section and the area assigned on the H&E section, respectively. Before enumeration of the FISH stained section, note the overall signal distribution (homogenous or heterogeneous) on the signal enumeration sheet. In case of heterogeneous distribution, note whether focal amplification or single cell amplification (mosaic) is present.

1) Homogenous signal distribution
In case the signal distribution is homogenous, enumerate the number of chromosome centromeres (green signals) and the number of \textit{HER2} genes (red signals) respectively, from 20 cells in 1-2 representative tumor areas.

2) Heterogeneous signal distribution
In case the signal distribution is heterogeneous, enumerate a total of 20 cells from selected areas as specified below:

A) If focal amplification exists, areas with amplified cells should be selected.
B) If mosaic distribution or amplified, polysomal and disomal cells are present, count in areas with amplified cells. Within these areas, not only amplified cells but also adjacent non-amplified cells should be counted for a total of 20 cells.

If possible, do not select overlapping areas.

Disregard staining of bacterial DNA
A number of specialized cells (mast cells and macrophages), present interspersed in the gastric tissue, exhibit a high level of staining by the \textit{HER2} probe due to presence of bacterial DNA. This results in highly red fluorescent cells that are clearly distinct from tumor cells with high \textit{HER2} gene amplification.

Signal enumeration: When an area has been selected for signal evaluation, begin analysis in one of the 20 adjacent chosen nuclei and then count in a cell-by-cell fashion only leaving out nuclei that do not meet the quality criteria. Locate the tumor within the context of the H&E stained slide and evaluate the same area on the FISH stained slide. Scan the entire stained slide 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 7).

- 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. The distance has to be at least equal to the diameter of one normal-sized signal in order to count two individual signals. When the distance between two signals is less than the diameter of a signal it is counted as one.
- In nuclei with high levels of \textit{HER2} gene amplification, the \textit{HER2} signals may be positioned very close to each other forming a cluster of signals. In these cases the number of \textit{HER2} signals cannot be counted, but must be estimated. Special attention must be paid to the green signals, as clusters of red \textit{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 color. Score only those nuclei with one or more FISH signals of each color.

**Signal counting guide**

<table>
<thead>
<tr>
<th></th>
<th><img src="image1" alt="Nuclei Image" /></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image2" alt="Nuclei Image" /></td>
<td>Do not count. Nuclei are overlapping, not all areas of nuclei are visible</td>
</tr>
<tr>
<td>2</td>
<td><img src="image3" alt="Nuclei Image" /></td>
<td>Two green signals, do not score nuclei with signals of only one color</td>
</tr>
<tr>
<td>3</td>
<td><img src="image4" alt="Nuclei Image" /></td>
<td>Count as 3 green and 12 red signals (cluster estimation)</td>
</tr>
<tr>
<td>4</td>
<td><img src="image5" alt="Nuclei Image" /></td>
<td>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</td>
</tr>
<tr>
<td>5</td>
<td><img src="image6" alt="Nuclei Image" /></td>
<td>Do not count (over- or underdigested nuclei). Missing signals in the centre of nuclei (donut-shaped nuclei).</td>
</tr>
<tr>
<td>6</td>
<td><img src="image7" alt="Nuclei Image" /></td>
<td>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</td>
</tr>
<tr>
<td>7</td>
<td><img src="image8" alt="Nuclei Image" /></td>
<td>Count as 1 green and 5 red signals</td>
</tr>
<tr>
<td>8</td>
<td><img src="image9" alt="Nuclei Image" /></td>
<td>Count as 3 green (1 green out of focus) and 3 red signals</td>
</tr>
<tr>
<td>9</td>
<td><img src="image10" alt="Nuclei Image" /></td>
<td>Cluster of red signals hiding green signals, check the green signals with a specific FITC filter, or do not count</td>
</tr>
</tbody>
</table>

Record counts in a table as shown in Appendices 5-6.

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

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 (39).

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 40 nuclei and calculate the ratio for the 40 nuclei. If the enumeration continues to be borderline, the result of the second evaluation is valid. If available, the immunohistochemical staining of HER2 should be included for better orientation during the second enumeration.

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 - Gastric

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 - Gastric

Background and clinical data
The clinical safety and efficacy of trastuzumab (Herceptin®) has been demonstrated in the BO18255 study (the ToGA trial “An open-label, randomized, multicenter, phase III study of trastuzumab in combination with cisplatin and a fluoropyrimidine (capecitabine or 5-Fluorouracil) (FC+H) versus chemotherapy (FC) alone as first-line therapy in patients with HER2 positive advanced gastric cancer”) (38). The study was designed as an open labeled, randomized, multicenter phase III study in HER2-positive patients with advanced gastric or gastroesophageal junction adenocarcinoma. In the BO18255 study HER2 positivity was defined as being either IHC-positive (3+) using HercepTest™ (Dako) and/or FISH positive (HER2/CEN-17 ≥ 2.0) using HER2 FISH pharmDx Kit (Dako). After inclusion in the study the patients were randomized to receive chemotherapy (5-FU or capecitabine and cisplatin) or chemotherapy plus trastuzumab.

The main efficacy outcome measure was overall survival (OS) analyzed by stratified log rank test. The final OS analysis based on 351 deaths was statistically significant (nominal significance level of 0.0193). An updated OS analysis was also conducted at one year after the final analysis. The median overall survival of 13.5 months on the Herceptin® plus chemotherapy arm was significantly longer compared to 11.0 month median overall survival on the chemotherapy alone arm. The efficacy results of both the final and the updated analyses are summarized in Table 9 and Figure .
Table 9. Overall Survival in Intention to Treat (ITT) Population.

<table>
<thead>
<tr>
<th></th>
<th>FC Arm N=296</th>
<th>FC + H Arm N=298</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Final Overall Survival</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. Deaths (%)</td>
<td>184 (62.2%)</td>
<td>167 (56.0%)</td>
</tr>
<tr>
<td>Median</td>
<td>11.0</td>
<td>13.5</td>
</tr>
<tr>
<td>95% CI (mos.)</td>
<td>(9.4, 12.5)</td>
<td>(11.7, 15.7)</td>
</tr>
<tr>
<td><strong>Hazard Ratio</strong></td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>95% CI</td>
<td>(0.60, 0.91)</td>
<td></td>
</tr>
<tr>
<td>p-value*, two-sided</td>
<td>0.0038*</td>
<td></td>
</tr>
</tbody>
</table>

|                      |              |                  |
| **Updated Overall Survival** |          |                  |
| No. Deaths (%)       | 227 (76.7%)  | 221 (74.2%)      |
| Median               | 11.7         | 13.1             |
| 95% CI (mos.)        | (10.3, 13.0) | (11.9, 15.1)     |
| **Hazard Ratio**     | 0.80         |                  |
| 95% CI               | (0.67, 0.97) |                  |

*Comparing with the nominal significance level of 0.0193

**Figure 7.** Updated Overall Survival in Patients with Metastatic Gastric Cancer
An exploratory analysis of OS based on gene amplification (FISH) and protein-overexpression (IHC) testing is summarized in Table 10.

Table 10. Exploratory Analyses by HER2 Status with Updated Overall Survival Results.

<table>
<thead>
<tr>
<th></th>
<th>FC N=296a</th>
<th>FC+H N=298b</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FISH+ / IHC 0, 1+ subgroup (N=133)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. Deaths / n (%)</td>
<td>57/71 (80.3%)</td>
<td>56/62 (90.3%)</td>
</tr>
<tr>
<td>Median OS Duration (mos.)</td>
<td>8.8 (6.4, 11.7)</td>
<td>8.3 (6.2, 10.7)</td>
</tr>
<tr>
<td>95% CI (mos.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hazard ratio (95% CI)</td>
<td>1.33 (0.92, 1.92)</td>
<td></td>
</tr>
<tr>
<td><strong>FISH+ / IHC2+ subgroup (N=160)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. Deaths / n (%)</td>
<td>65/80 (81%)</td>
<td>64/80 (80%)</td>
</tr>
<tr>
<td>Median OS Duration (mos.)</td>
<td>10.8 (6.8, 12.8)</td>
<td>12.3 (9.5, 15.7)</td>
</tr>
<tr>
<td>95% CI (mos.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hazard ratio (95% CI)</td>
<td>0.78 (0.55, 1.10)</td>
<td></td>
</tr>
<tr>
<td><strong>FISH+ or FISH-/IHC3+c subgroup (N=294)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. Deaths / n (%)</td>
<td>104/143 (73%)</td>
<td>96/151 (64%)</td>
</tr>
<tr>
<td>Median OS Duration (mos.)</td>
<td>13.2 (11.5, 15.2)</td>
<td>18.0 (15.5, 21.2)</td>
</tr>
<tr>
<td>95% CI (mos.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hazard ratio (95% CI)</td>
<td>0.66 (0.5, 0.87)</td>
<td></td>
</tr>
</tbody>
</table>

Median survival was estimated from Kaplan-Meier curves.

- Two patients on FC arm who were FISH+ but IHC status unknown were excluded from the analyses.
- Five patients on Herceptin® arm who were FISH+ but IHC status unknown were excluded from the analyses.
- Includes 6 patients on chemotherapy arm, 10 patients on Herceptin® arm with FISH-, IHC3+ and 8 patients on chemotherapy arm, 8 patients on Herceptin® arm with FISH status unknown, IHC3+.

Hybridization efficiency
Hybridization efficiency of HER2 IQFISH pharmDx was investigated as part of the reproducibility study. From the total 180 formalin-fixed, paraffin-embedded tissue sections tested at the three study sites all 180 could be enumerated in accordance with product guidelines. Thus, the hybridization efficiency was 100%.

Analytical sensitivity
The analytical sensitivity of the HER2/CEN-17 IQFISH Probe Mix was investigated using 18 gastric cancer adenocarcinoma specimens. The ratio between the number of HER2 signals and CEN-17 signals was calculated based on a counting of 20 nuclei from normal cells surrounding the tumor. The HER2/CEN-17 ratio for the 18 gastric cancer adenocarcinoma specimens was between 0.95 and 1.06.

Analytical specificity
The HER2 DNA probes in the HER2/CEN-17 IQFISH 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 IQFISH 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 Dako QC procedures. A total of 275 metaphase spreads with distinct signals were evaluated for specific hybridization of the HER2
DNA and CEN-17 PNA probe mixes. In all 275 cases, the hybridization was specific for chromosome 17. No cross-hybridization to loci on other chromosomes was observed in any of the 275 cases. Probe specificity was then 100% (275 of 275).

To measure the assay's ability to solely identify the target substances HER2 and CEN-17 without interference from other substances studies were performed on gastric adenocarcinoma specimens using Vial 3 containing the hybridization buffer but without the probe mix. A total of 18 specimens were evaluated for presence of signals not related to the probe mix. No detection of other chromosome targets or interference with closely related substances was observed in any of the 18 specimens.

**Robustness studies**

The robustness of the HER2 IQFISH pharmDx assay was tested by varying pre-treatment time, temperature, and methods for heating of pre-treatment buffer (microwave oven or water bath), pepsin incubation time and method (RTU pepsin or immersion), denaturation temperature and time, hybridization time, and stringent wash time and temperature.

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

- Pre-treatment Method A) Water bath for 10 minutes combined with each of the temperatures 95 °C, 95-99 °C, and 99 °C together with 9, 10 and 11 minutes at 95-99 °C.
- Pretreatment Method B) Microwave oven for 9, 10, and 11 minutes at > 95 °C.
- Pepsin digestion Method A) with incubation times of 5, 10, and 15 minutes at room temperature (20-25 °C).
- Pepsin digestion Method B) with incubation times of 3, 4, and 5 minutes at 37 °C.
- Pepsin digestion Method C) with incubation times of 20, 25, and 30 minutes combined with each of the temperatures 35, 37, and 39 °C.
- Denaturation for 10 minutes combined with each of the temperatures 65, 66, and 67 °C together with 9, 10, and 11 minutes at 66 °C.
- Hybridization times of 60, 90, and 120 minutes at 45 °C.
- Stringency wash for 10 minutes combined with each of the temperatures 61, 63, and 65 °C together with 9, 10, and 11 minutes at 63 °C.

**Note:** For the robustness tests only one parameter in the staining procedure was changed at a time while all other parameters were kept constant. It is recommended to adhere to the time and temperatures indicated in the staining procedure.

The staining procedure for HER2 IQFISH pharmDx offers protocol variables for heat pre-treatment, pepsin digestion and hybridization time. Each unique combinatorial option has been validated with regard to HER2 status. Validation was performed on 10 FFPE specimens of human gastric and gastroesophageal junction adenocarcinoma for each of the 12 possible combinatorial options. HER2 FISH pharmDx Kit (K5331) was used as reference. HER2/CEN-17 ratio for each individual specimen is shown in Figure 8. Cross tabulations between the 12 tests and the reference staining showed overall agreement of 100% (10/10) with lower and upper two-tailed 95% confidence limits at 78.3% and 100%, respectively. Kappa value was 1.00 and McNemars test showed absence of bias (two-tailed p-value of 1.00).
Figure 8. Individual HER2/CEN-17 ratios for each human gastric adenocarcinoma specimen obtained by staining performed with the 12 combinatorial staining protocol variations possible with the HER2 IQFISH pharmDx (K5731) (Test 1-12) and the HER2 FISH pharmDx Kit (K5331) reference (R). The horizontal line illustrates the cut-off value of 2.0.

Repeatability
The repeatability of the HER2/CEN-17 ratio was investigated with the HER2 IQFISH pharmDx assay using consecutive sections from nine different gastric adenocarcinoma specimens with either non-amplified or amplified HER2 gene status. Triplicate sections of each specimen were tested in the same run. The average coefficient of variance was 3.5% for non-amplified specimens (range from 1% to 5%) and 2.8% for amplified specimens (range 1% to 5%).

Repeatability on consecutive sections of gastric adenocarcinoma specimens with different thickness (2, 3, 4, 5, 6, and 7 µm) was tested with HER2 IQFISH pharmDx. The average coefficient of variance of the HER2/CEN-17 ratio was 4.5% (range from 3% to 6%). i.e. in the same range as for tissue of equal thickness.

Reproducibility
The day-to-day, observer-to-observer, and site-to-site reproducibility was tested using HER2 IQFISH pharmDx. The study was designed as a three-site, blinded, randomized, comparative study of HER2/CEN-17 ratios using sections from 12 different formalin-fixed, paraffin-embedded gastric cancer specimens including gastroesophageal junction (GEJ) with different levels of HER2 gene amplification. The 12 specimens had been tested with HercepTest™ and represented three specimens with HER2 IHC 0/1+, three specimens with HER2 IHC 2+, three specimens with HER2 IHC 3+, and three specimens with pre-determined HER2/CEN-17 FISH ratio between 1.5 and 2.5 obtained using HER2 IQFISH pharmDx. The specimens were stained and analyzed at three different study sites in USA and Europe. Each specimen was stained five times on five non-consecutive days and counted by two observers at each of the three study sites. A total of 180 stainings and 360 observations were made for the entire study. Variations in ratios between the different sites, days, and observers are illustrated in Figure . Data were analyzed using Box-Cox transformation. Estimated coefficient of variation for the non-amplified (180 observations) and amplified (180 observations) group of specimens was calculated to 0.145 and 0.234, respectively. Estimated coefficient of variation of the IHC 2+ category (90 observations) of specimens was calculated to 0.234, whereas the estimated coefficient of variation for the entire study was calculated to 0.229.
Figure 9. HER2/CEN-17 ratios obtained in the reproducibility study on HER2 IQFISH pharmDx including site-to-site, observer-to-observer, and day-to-day reproducibility.

Furthermore, the HER2 IQFISH pharmDx assay was tested for lot-to-lot reproducibility using three different lots of HER2 IQFISH pharmDx. Reproducibility was tested on nine different gastric adenocarcinoma specimens with either non-amplified or amplified HER2 gene status. The average coefficient of variation for lot-to-lot reproducibility was 3.8% for non-amplified specimens (range from 2% to 7%) and 1.8% for amplified specimens (range from 1% to 3%). Gastric adenocarcinoma specimens consisted of 78.8% resection and 22.2% biopsy specimens. 55.6% of the specimens were obtained from stomach and 44.4% were from gastroesophageal junction.

Comparison study
The clinical utility of HER2 IQFISH pharmDx (Code K5731) was investigated in a comparative study with HER2 FISH pharmDx Kit (Code K5331). The study included 139 gastric cancer specimens consisting of the different gastric adenocarcinoma tissue types, i.e. stomach or gastro-esophageal junction and resections or biopsies with homogeneous or heterogeneous (focal or mosaic) signal distribution as shown in Table 11.

Table 11. Distribution of specimens in gastric cancer type (stomach or GEJ), signal distribution category (homogeneous or heterogeneous), and tissue type (resection or biopsy).

<table>
<thead>
<tr>
<th>Gastric cancer type</th>
<th>Number of specimens</th>
<th>Signal distribution</th>
<th>Number of specimens</th>
<th>Tissue type</th>
<th>Number of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>91</td>
<td>Homogeneous</td>
<td>38</td>
<td>Resection</td>
<td>126</td>
</tr>
<tr>
<td>Gastro-esophageal junction (GEJ)</td>
<td>48</td>
<td>Heterogeneous - focal</td>
<td>51</td>
<td>Biopsy</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heterogeneous - mosaic</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>139</td>
<td>Total</td>
<td>139</td>
<td>Total</td>
<td>139</td>
</tr>
</tbody>
</table>
The specimens consisted of 77 HER2 non-amplified and 62 HER2 amplified cases with known HercepTest™ score as shown in Table 12.

Table 12. Distribution of specimens based on HercepTest™ score and HER2 FISH score obtained with HER2 FISH pharmDx Kit (K5331).

<table>
<thead>
<tr>
<th>HercepTest™ staining score</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>23</td>
<td>55</td>
<td>41</td>
<td>139</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HER2 FISH status</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplified</td>
<td>0</td>
<td>3</td>
<td>19</td>
<td>41</td>
<td>62</td>
</tr>
<tr>
<td>Non-amplified</td>
<td>20</td>
<td>20</td>
<td>36</td>
<td>0</td>
<td>77</td>
</tr>
<tr>
<td>Total FISH tested samples</td>
<td>20</td>
<td>23</td>
<td>55</td>
<td>41</td>
<td>139</td>
</tr>
</tbody>
</table>

Results of the cross tabulation of HER2 status obtained by the two assays with calculation of overall percent agreement (OPA), positive percent agreement (PPA), and negative percent agreement (NPA) with 95% confidence intervals are shown in Table 13. Correlation between the two assays is shown in Figure. The Kappa value was 0.97 with lower and upper limits for the 95% confidence interval at 0.93 and 1.00. The p-value for McNemars test was 1.00 indicating absence of bias between the two assays.

Table 13. Cross tabulation of HER2 gene status obtained using HER2 IQFISH pharmDx (K5731) and HER2 FISH pharmDx Kit (K5331).

<table>
<thead>
<tr>
<th>HER2 gene status (K5731)</th>
<th>Non-amplified</th>
<th>Amplified</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-amplified</td>
<td>75</td>
<td>1</td>
<td>76</td>
</tr>
<tr>
<td>Amplified</td>
<td>1</td>
<td>62</td>
<td>63</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
<td>63</td>
<td>139</td>
</tr>
</tbody>
</table>

OPA: 98.6% [95.5%; 99.7%]
PPA: 98.4% [92.8%; 99.8%]
NPA: 98.7% [94.0%; 99.9%]
Figure 10. Correlation between HER2 FISH pharmDx Kit (K5331) and HER2 IQFISH pharmDx (K5731) on 139 gastric cancer specimens. The main figure includes specimens with HER2/CEN-17 ratio ≤ 17. The small inserted figure at the top of the main figure includes specimens with both HER2/CEN-17 ratio ≤ 17 and ratio > 17.

Two discrepant cases were found as shown in Table 14. Re-examination of the specimens showed that the tissues were heterogeneous (mosaic) and contained amplified areas with a low copy number of the HER2 gene. Disagreement in HER2 status for these two specimens is therefore likely to be a result of heterogeneous tissues with HER2/CEN-17 ratios close to the borderline region (1.8-2.2).
Table 14. Specimens with disagreement in HER2 status obtained by use of HER2 FISH pharmDx Kit (K5331) and HER2 IQFISH pharmDx (K5731).

<table>
<thead>
<tr>
<th>ID #</th>
<th>HercepTest™ score</th>
<th>HER2/CEN-17 ratio (K5331)</th>
<th>HER2/CEN-17 ratio (K5731)</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>1+ (focal 2+)</td>
<td>2.35</td>
<td>1.78</td>
</tr>
<tr>
<td>63</td>
<td>1+ (focal 2+)</td>
<td>1.68</td>
<td>2.40</td>
</tr>
</tbody>
</table>

Cross tabulation of HER2 status was also performed for the IHC 2+ subset of the gastric cancer specimens (n = 55), as shown in Table 15. The Kappa value was 1 and McNemars test showed no bias between the two assays (p-value of 1.00).

Table 15. Cross tabulation of HER2 gene status obtained using HER2 IQFISH pharmDx (K5731) and HER2 FISH pharmDx Kit (K5331) for the IHC 2+ subset of specimens.

<table>
<thead>
<tr>
<th>HER2 gene status (K5331)</th>
<th>Total</th>
<th>Non-amplified</th>
<th>Amplified</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER2 gene status (K5731)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-amplified</td>
<td>36</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>Amplified</td>
<td>0</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>19</td>
<td>55</td>
</tr>
</tbody>
</table>

OPA: 100% [95.6%; 100%]  
PPA: 100% [87.8%; 100%]  
NPA: 100% [93.3%; 100%]
## Troubleshooting - Gastric Cancer

<table>
<thead>
<tr>
<th>Problem</th>
<th>Probable Cause</th>
<th>Suggested Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. No signals or weak signals</td>
<td>1a. Kit has been exposed to high temperatures during transport or storage</td>
<td>1a. Check storage conditions. Ensure that dry ice was present when the consignment was received. Ensure that vial 3 has been stored at -18 °C in the dark. Ensure that vials 2A and 5 have been stored at maximum 2-8 °C, and that vials have been stored in the dark.</td>
</tr>
<tr>
<td></td>
<td>1b. Microscope not functioning properly</td>
<td>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 7). In case of doubt, please contact your local microscope vendor.</td>
</tr>
<tr>
<td></td>
<td>- Inappropriate filter set</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Improper lamp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Mercury lamp too old</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Dirty and/or cracked collector lenses</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Unsuitable immersion oil</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1c. Faded signals</td>
<td>1c. Avoid long microscopic examination and minimize exposure to strong light sources.</td>
</tr>
<tr>
<td></td>
<td>1d. Pre-treatment conditions incorrect</td>
<td>1d. Ensure that the recommended pre-treatment temperature and time are used.</td>
</tr>
<tr>
<td></td>
<td>1e. Evaporation of Probe Mix during hybridization</td>
<td>1e. Ensure sufficient humidity in the hybridization chamber.</td>
</tr>
<tr>
<td>2. No green signals</td>
<td>2a. Stringent wash conditions incorrect</td>
<td>2a. Ensure that the recommended stringent wash temperature and time are used, and that coverslips are removed before performing stringent wash.</td>
</tr>
<tr>
<td>3. No red signals</td>
<td>3a. Pre-treatment conditions incorrect</td>
<td>3a. Ensure that the recommended pre-treatment temperature and time are used.</td>
</tr>
<tr>
<td>4. Areas without signal</td>
<td>4a. Probe volume too small</td>
<td>4a. Ensure that the probe volume is large enough to cover the area under the coverslip.</td>
</tr>
<tr>
<td></td>
<td>4b. Air bubbles caught during Probe Mix application or mounting</td>
<td>4b. Avoid air bubbles. If observed, gently tap them away using forceps.</td>
</tr>
<tr>
<td>Problem</td>
<td>Probable Cause</td>
<td>Suggested Action</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>-----------------------------------------------------</td>
<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>5. Excessive background staining</td>
<td>5a. Inappropriate tissue fixation.</td>
<td>5a. Ensure that only formalin-fixed, paraffin-embedded tissue sections are used.</td>
</tr>
<tr>
<td></td>
<td>5b. Paraffin incompletely removed</td>
<td>5b. Follow the deparaffinization and rehydration procedures outlined in Section B.2.</td>
</tr>
<tr>
<td></td>
<td>5c. Stringent wash temperature too low</td>
<td>5c. Ensure that the stringent wash temperature is 63 (±2) °C.</td>
</tr>
<tr>
<td></td>
<td>5d. Prolonged exposure of hybridized section to strong light</td>
<td>5d. Avoid long microscopic examination and minimize exposure to strong light.</td>
</tr>
<tr>
<td>6. Poor tissue morphology</td>
<td>6a. Incorrect Pepsin treatment</td>
<td>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.</td>
</tr>
<tr>
<td></td>
<td>6b. Incorrect pre-treatment conditions may result in unclear or cloudy appearance</td>
<td>6b. Ensure that the recommended pre-treatment temperature and time are used.</td>
</tr>
<tr>
<td></td>
<td>6c. Too long Pepsin treatment or very thin section thickness may cause ghost cells or donut cells to appear.</td>
<td>6c. Shorten the Pepsin incubation time. See section B.3, step 2. Ensure that the section thickness is 3-6 µm.</td>
</tr>
<tr>
<td>7. High level of green auto fluorescence on slide including areas without FFPE tissue</td>
<td>7. Use of old glass slides</td>
<td>7. Ensure that the coated glass slides (Dako Silanized Slides, Code S3003, or poly-L-lysine-coated slides) have not passed expiry date.</td>
</tr>
</tbody>
</table>

**NOTE:** If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please call Dako Technical Services for further assistance.
Appendix 4 – Gastric

**HER2 IQFISH pharmDx, Code K5731**

Protocol Checklist

<table>
<thead>
<tr>
<th>Staining Run Log ID: ______________________</th>
</tr>
</thead>
</table>

- **Date of the run:** ______________________
- **HER2 IQFISH pharmDx, K5731 Lot:** ______________________
- **Specimen ID:** ______________________
- **Equipment ID:** ______________________
- **Date of dilution/expiration of the 1 x Wash Buffer (Vial 6 diluted 1:20):** __________ / __________

<table>
<thead>
<tr>
<th>Tissue fixed in neutral buffered formalin</th>
<th>Yes ☐</th>
<th>No ☐</th>
</tr>
</thead>
</table>

### Step 1: Pre-Treatment

<table>
<thead>
<tr>
<th>Date of dilution/expiration of the Pre-Treatment Solution (Vial 1 diluted 1:20)</th>
<th>/</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured temperature of Pre-Treatment Solution (95-99 °C) if water bath is used for heating</td>
<td>°C</td>
</tr>
<tr>
<td>Pre-treatment (10 minutes), and cooling (15 minutes)</td>
<td></td>
</tr>
<tr>
<td>Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes)</td>
<td></td>
</tr>
</tbody>
</table>

### Step 2: Pepsin

<table>
<thead>
<tr>
<th>Duration of Pepsin (Vial 2) treatment at 37 °C or Room temperature or</th>
<th>Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of Pepsin (Vial 2) treatment at room temperature or</td>
<td>Minutes</td>
</tr>
<tr>
<td>Duration of Pepsin immersion at 37 (±2) °C</td>
<td>Minutes</td>
</tr>
<tr>
<td>Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes)</td>
<td></td>
</tr>
<tr>
<td>Dehydrate slides (3 x 2 minutes) in graded series of ethanol and air dry</td>
<td></td>
</tr>
</tbody>
</table>

### Step 3: HER2/CEN-17 IQISH Probe Mix

<table>
<thead>
<tr>
<th>Apply Probe Mix (Vial 3), coverslip and seal with Coverslip Sealant</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured denaturation temperature (66 ±1 °C)</td>
<td>°C</td>
</tr>
<tr>
<td>Denaturation for 10 minutes</td>
<td></td>
</tr>
<tr>
<td>Measured hybridization temperature (45 °C)</td>
<td>°C</td>
</tr>
<tr>
<td>Hybridization time (60 to 120 minutes)</td>
<td>Minutes</td>
</tr>
</tbody>
</table>

### Step 4: Stringent Wash

<table>
<thead>
<tr>
<th>Date of dilution/expiration of the Stringent Wash Buffer (Vial 4 diluted 1:20)</th>
<th>/</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured temperature of Stringent Wash Buffer (63 ±2 °C)</td>
<td>°C</td>
</tr>
<tr>
<td>Stringent wash (10 minutes) after removing the coverslips</td>
<td></td>
</tr>
<tr>
<td>Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes)</td>
<td></td>
</tr>
<tr>
<td>Dehydrate slides (3 x 2 minutes) in graded series of ethanol and air dry</td>
<td></td>
</tr>
</tbody>
</table>

### Step 5: Mounting

<table>
<thead>
<tr>
<th>Apply 15 μL of Fluorescence Mounting Medium (Vial 5) and coverslip</th>
<th></th>
</tr>
</thead>
</table>

Comments: ________________________________________________________

____________________________________________________

Date and signature, Technician: ____________________________________
Appendix 5 - Gastric

HER2 IQFISH pharmDx, Code K5731

Scoring Scheme

Date of the run: __________________ Staining Run Log ID: ________________
HER2 IQFISH pharmDx, K5731 Lot: ________ Specimen ID: _____________

Characterization of signal distribution in tissue:

Homogeneous: □

Heterogeneous – Focal: □ or Heterogeneous – Mosaic: □

<table>
<thead>
<tr>
<th>Count signals in 20 nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus No.</td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>9</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>Total (1-10)</td>
</tr>
</tbody>
</table>

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 40 nuclei and recalculate the ratio for the 40 nuclei (refer to recount scoring scheme, Appendix 6).

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

<table>
<thead>
<tr>
<th></th>
<th>HER2</th>
<th>CEN-17</th>
<th>HER2/CEN-17 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total score (1-20)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

☐ 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.
Appendix 6 - Gastric

HER2 IQFISH pharmDx, Code K5731
Recount Scoring Scheme

HER2 IQFISH pharmDx, K5731 lot: __________ Staining Run Log ID: __________
Date of the run: __________________________ Specimen ID: _______________

<table>
<thead>
<tr>
<th>Nuclei no.</th>
<th>Red HER2</th>
<th>Green CEN-17</th>
<th>Nuclei no.</th>
<th>Red HER2</th>
<th>Green CEN-17</th>
<th>Nuclei no.</th>
<th>Red HER2</th>
<th>Green CEN-17</th>
<th>Nuclei no.</th>
<th>Red HER2</th>
<th>Green CEN-17</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>21</td>
<td>11</td>
<td>21</td>
<td>31</td>
<td>31</td>
<td>21</td>
<td>31</td>
<td>31</td>
<td>21</td>
<td>31</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>22</td>
<td>12</td>
<td>22</td>
<td>32</td>
<td>32</td>
<td>22</td>
<td>32</td>
<td>32</td>
<td>22</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>23</td>
<td>13</td>
<td>23</td>
<td>33</td>
<td>33</td>
<td>23</td>
<td>33</td>
<td>33</td>
<td>23</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>24</td>
<td>14</td>
<td>24</td>
<td>34</td>
<td>34</td>
<td>24</td>
<td>34</td>
<td>34</td>
<td>24</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>25</td>
<td>15</td>
<td>25</td>
<td>35</td>
<td>35</td>
<td>25</td>
<td>35</td>
<td>35</td>
<td>25</td>
<td>35</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>26</td>
<td>16</td>
<td>26</td>
<td>36</td>
<td>36</td>
<td>26</td>
<td>36</td>
<td>36</td>
<td>26</td>
<td>36</td>
</tr>
<tr>
<td>7</td>
<td>17</td>
<td>27</td>
<td>17</td>
<td>27</td>
<td>37</td>
<td>37</td>
<td>27</td>
<td>37</td>
<td>37</td>
<td>27</td>
<td>37</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>28</td>
<td>18</td>
<td>28</td>
<td>38</td>
<td>38</td>
<td>28</td>
<td>38</td>
<td>38</td>
<td>28</td>
<td>38</td>
</tr>
<tr>
<td>9</td>
<td>19</td>
<td>29</td>
<td>19</td>
<td>29</td>
<td>39</td>
<td>39</td>
<td>29</td>
<td>39</td>
<td>39</td>
<td>29</td>
<td>39</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>30</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>40</td>
<td>30</td>
<td>40</td>
<td>40</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>Total 1-10</td>
<td>Total 11-20</td>
<td>Total 21-30</td>
<td>Total 21-30</td>
<td>Total 31-40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For determination of the HER2/CEN-17 ratio, count the number of HER2 signals and the number of CEN-17 signals in the same 40 nuclei and divide the total number of HER2 signals by the total number of CEN-17 signals. Report Total Score from the 1-40 nuclei in the table below.

<table>
<thead>
<tr>
<th>HER2 FISH</th>
<th>HER2</th>
<th>CEN-17</th>
<th>HER2/CEN-17 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOTAL SCORE (1-40)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- 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.
Appendix 7 - Gastric

HER2 IQFISH pharmDx, Code K5731
Fluorescence Microscope Specifications

Dako recommends the following equipment for use with HER2 IQFISH pharmDx, K5731:

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. Dako recommends the use of a specific DAPI filter in combination with a high quality Texas Red/FITC double filter.
   - DAPI filter.
   - Texas Red/FITC double filter.
   - Texas Red and FITC single filters can be used for confirmation.

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Excitation Wavelength</th>
<th>Emission Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC</td>
<td>495 nm</td>
<td>520 nm</td>
</tr>
<tr>
<td>Texas Red</td>
<td>596 nm</td>
<td>615 nm</td>
</tr>
</tbody>
</table>

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 Dako 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.
References


## Explanation of symbols

<table>
<thead>
<tr>
<th>REF</th>
<th>Catalogue number</th>
<th>Keep away from sunlight (consult storage section)</th>
<th>Manufacturer</th>
<th>GHS hazard pictogram (consult precautions section)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVD</td>
<td>In vitro diagnostic medical device</td>
<td>Contains sufficient for &lt;n&gt; tests</td>
<td>GHS hazard pictogram (consult precautions section)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Consult instructions for use</td>
<td>LOT</td>
<td>Batch code</td>
<td>GHS hazard pictogram (consult precautions section)</td>
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<tr>
<td></td>
<td>Temperature limitation</td>
<td></td>
<td>Use by</td>
<td>GHS hazard pictogram (consult precautions section)</td>
</tr>
</tbody>
</table>

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