



HER2 CISH pharmDx™ Kit

Code SK109

1st edition

HER2 CISH pharmDx™ Kit is a direct dual-color chromogenic in situ hybridization (CISH) assay designed to quantitatively determine *HER2* gene amplification in formalin-fixed, paraffin-embedded (FFPE) breast cancer tissue specimens. *HER2* CISH pharmDx™ Kit generates red (*HER2*) and blue (CEN-17) chromogenic signals on the same tissue section for evaluation by bright field microscopy.

The kit contains reagents sufficient for 20 tests.

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Intended Use

For in vitro diagnostic use

HER2 CISH pharmDx™ Kit is intended for dual-color chromogenic visualization of signals achieved with directly labeled in situ hybridization probes targeting the *HER2* gene and centromeric region of chromosome 17. The kit is designed to quantitatively determine *HER2* gene status in formalin-fixed, paraffin-embedded breast cancer tissue specimens. Red and blue chromogenic signals are generated on the same tissue section for evaluation under bright field microscopy. The CISH procedure is automated using Dako Autostainer instruments.

HER2 CISH pharmDx™ Kit is indicated as an aid in the assessment of patients for whom Herceptin™ (trastuzumab) treatment is being considered. Results from the *HER2* CISH pharmDx™ Kit are intended for use as an adjunct to the clinicopathologic information currently used for estimating prognosis in stage II, node-positive breast cancer patients.

Summary and Explanation

The human *HER2* gene (also known as *ERBB2* or *NEU*) is located on chromosome 17 and encodes the HER2 protein or p185^{HER2}. The HER2 protein is a membrane receptor tyrosine kinase with homology to the epidermal growth factor receptor (EGFR or HER1) (1-2). The *HER2* gene is present in 2 copies in all normal diploid cells.

In a fraction of patients with breast cancer, the *HER2* gene is amplified as part of the process of malignant transformation and 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 15-20% of breast cancers (10). This upregulation is associated with poor prognosis, increased risk of recurrence, and lower overall 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).

Principle of Procedure

HER2 CISH pharmDx™ Kit contains all reagents required to complete a CISH procedure for formalin-fixed, paraffin-embedded (FFPE) tissue section specimens (except hematoxylin for counterstaining).

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 either at room temperature or at 37 °C. Optimal Pepsin incubation time depends on the fixation history of the tissue and should be determined by the user but 8 minutes at room temperature or 2 minutes at 37°C will be suitable for most specimens. 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) (13) and DNA technology. The Probe Mix consists of a mixture of Texas Red-labeled DNA probes covering a 218 kb region including the *HER2* gene on chromosome 17, and a mixture of fluorescein-labeled PNA probes targeted at the centromeric region of chromosome 17 (CEN-17). The specific hybridization to the two targets results in formation of a distinct red fluorescent signal at each *HER2* gene locus and a distinct green fluorescent signal at each chromosome 17 centromere. To diminish background staining, the Probe Mix also contains unlabelled PNA blocking probes. After a stringent wash and rinse in two different wash buffers the fluorescent probe signals are converted into chromogenic signals by an immunohistochemical staining procedure. The first step is to block endogenous peroxidase with ready-to-use Peroxidase Block, followed by incubation with ready-to-use CISH

Antibody Mix, comprised of anti-FITC conjugated with horseradish peroxidase (HRP) and anti-Texas Red conjugated with alkaline phosphatase (AP). Next, the tissue specimens are incubated with Red Chromogen solution followed by incubation with Blue Chromogen solution. The enzymatic conversion of the added chromogens results in formation of visible red and blue end-products at the antigen site (FISH probes). Thus, the red fluorescent signals are converted to red, chromogenic signals and the green fluorescent signals are converted to blue, chromogenic signals. The specimens are then counterstained and coverslipped. Results are evaluated using a bright field microscope.

Using a bright field microscope, the invasive component of the tumor cells are located, and enumeration of the red (*HER2*) and blue (CEN-17) signals is conducted. Then the *HER2*/CEN-17 ratio is calculated. Normal cells within the analyzed tissue section serve as an internal positive control of pre-treatment and hybridization efficiency. For details see the Interpretation of Staining section.

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

Reagents

Materials provided

The materials listed below are sufficient for 20 tests (a test is defined as one 22 mm x 22 mm target area). The number of tests is based on the use of 5-8 drops (250 µL per slide) of Vial 2, 10 µL per slide of Vial 3, 400 µL per slide of vial 7 and 8, and 400 µL of the Red Chromogen (Vial 11) and Blue Chromogen (Vial 12) diluted in their respective substrate buffers (Vial 9 and 10). The kit provides materials sufficient for 20 tests in 10 individual staining runs. For staining on Dako Autostainer Link platform, the kit provides materials sufficient for 20 tests in 5 individual staining runs.

The *HER2* CISH pharmDx™ Kit is shipped as two separate items; SK109 Vial 2 (Pepsin) is shipped in a separate box and the remaining reagents in one kit box. SK109 Vial 2 (Pepsin) is shipped on dry ice and the kit box is shipped on cooling packs. **To ensure that Vial 2 (Pepsin) has not been exposed to high temperatures during transport dry ice should still be present in shipment upon receipt.**

Vial 1

PRE-TREATMENT SOLUTION (20x)

Pre-Treatment Solution (20x)

150 mL, concentrated 20x
MES (2-[*N*-morpholino]ethanesulphonic acid) buffer.

Vial 2

PEPSIN

Pepsin

7.5 mL, ready-to-use
Pepsin solution, pH 2.0; contains stabilizer and an antimicrobial agent.

Vial 3

**HER2/CEN-17
PROBE MIX**

HER2/CEN-17 Probe Mix

200 µL, ready-to-use
Mix of Texas Red-labelled *HER2* DNA probes and fluorescein-labelled CEN-17 PNA probes; supplied in hybridization buffer with 45% formamide, stabilizer, and unlabelled PNA blocking probes.

- Vial 4** STRINGENT WASH BUFFER (20x)
Stringent Wash Buffer (20x)
150 mL, concentrated 20x
SSC (saline-sodium citrate) buffer with detergent.
- Vial 5** WASH BUFFER 1 (20x)
Wash Buffer 1 (20x)
500 mL, concentrated 20x
Tris/HCl buffer.
- Vial 6** WASH BUFFER 2 (10x)
Wash Buffer 2 (10x)
1 L, concentrated 10x
Tris-buffered saline solution with 0.05% Tween 20.
- Vial 7** PEROXIDASE BLOCK
Peroxidase Block
9 mL, ready-to-use. 3% hydrogen peroxidase containing
15 mmol/L sodium azid (NaN_3).
- Vial 8** CISH ANTIBODY MIX
CISH Antibody Mix
9 mL, ready-to-use.
Mix of horseradish peroxidase-conjugated antibody to
fluorescein and alkaline phosphatase-conjugated antibody
to Texas Red; Supplied in 50 mmol/L Tris buffer with
stabilizer, preservative, pH 7.5.
- Vial 9** RED SUBSTRATE BUFFER
Red Substrate Buffer
12 mL, substrate buffer for dilution of Red Chromogen.
- Vial 10** BLUE SUBSTRATE BUFFER
Blue Substrate Buffer
12 mL, substrate buffer for dilution of Blue Chromogen.
- Vial 11** RED CHROMOGEN
Red Chromogen
120 μL , to be diluted in Red Substrate Buffer (Vial 9).
- Vial 12** BLUE CHROMOGEN
Blue Chromogen
2 x 550 μL , to be diluted in Blue Substrate Buffer (Vial 10).
- Vial 13** Tissue-Clear®-BASED
MOUNTING MEDIUM
Tissue-Clear®-Based Mounting Medium
5 mL, permanent mounting media without alcohol or xylene

COVERSLIP SEALANT

Coverslip Sealant

1 tube, ready-to-use

Solution for removable sealing of coverslips.



User-Fillable Reagent Bottles

5 mL Capacity, 6 Bottles*

12 mL Capacity, 2 Bottles**

For use on Dako Autostainer Link platform

* To run more than 4 separate stains, additional bottles will be needed.

** The 12 mL bottles are needed if all 20 tests are to be processed in one staining run

Materials required but not provided

Laboratory reagents:

Distilled or deionized water

Ethanol, 96%

Xylene or xylene substitutes

Dako Hematoxylin, Code S3301

User-fillable reagent bottles: 12 mL (Code SK201) or 5 mL (Code SK200) for diluted hematoxylin)

Laboratory equipment:

Lintless tissue

Adjustable pipettes

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

Calibrated surface thermometer (range 37-100 °C)*

Coverslips (22 mm x 22 mm for 10 µL hybridization and ex. 50 mm x 24 mm for mounting)

Forceps

Fume hood

Dako Hybridizer (Code S2450 or S2451)* + Humidity Control Strips (Code S2452)*

Slides, Dako Silanized Slides, Code S3003, or poly-L-lysine-coated slides (see Specimen Preparation)

Staining jars or baths

Whirl mixer (or equivalent) and table centrifuge

Timer (capable of 2-30 minute intervals)

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

Microwave oven with sensing capability if pre-treatment is performed using microwave oven (see B3. Staining protocol, Step 1: Pre-treatment, Method B)

Bright field microscope

* Heating block or hybridization oven for denaturation (82 (±2) °C) and hybridization (45 (±2) °C) together with a humid chamber can be used as an alternative to Dako Hybridizer.

Extra materials required for automated staining on Dako Autostainer/Autostainer Plus instruments include the following:

Autostainer Reagent Vials (Code S3425)

Precautions

1. For in vitro diagnostic use
2. For professional users.
3. Vial 1, Pre-Treatment Solution (20x), contains 1-<20% 2-morpholinoethanesulphonic acid;
Vial 2, Pepsin, contains 5-10% propan-2-ol;
Vial 4, Stringent Wash Buffer (20x), contains 1-<5% octoxinol
Vial 5, Wash Buffer 1 (20x), contains 1-<20% trometamol.
Vial 7, Peroxidase Block, contains sodium azide
At product concentrations these substances do not require hazard labeling. Material Safety Data Sheets (MSDSs) are available for professional users on request.
4. Vial 2, Pepsin, contains pepsin A that may cause an allergic reaction.
5. Vial 3, *HER2/CEN-17* Probe Mix contains 45% formamide and is labeled:
Toxic.
R61 May cause harm to the unborn child.
S45 In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).
S53 Avoid exposure – obtain special instructions before use.
S60 This material and/or its container must be disposed of as hazardous waste.
As a general rule, persons under 18 years of age are not allowed to work with this product. Users must be carefully instructed in the proper working procedure, the dangerous properties of the product and the necessary safety instructions.
Please refer to the Material Safety Data Sheet (MSDS) for additional information.
6. Coverslip Sealant contains 60-100% naphtha (petroleum), hydrotreated light, and is labeled:
Extremely flammable.
Dangerous for the environment.
R11 Highly flammable.
R51/53 Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.
S9 Keep container in a well-ventilated place.
S16 Keep away from sources of ignition – No smoking.
S35 This material and its container must be disposed of in a safe way.
S57 Use appropriate container to avoid environmental contamination.
S61 Avoid release to the environment. Refer to special instructions/safety data sheets.
Please refer to the Material Safety Data Sheet (MSDS) for additional information.
7. Vial 7, Peroxidase block, contains sodium azide (NaN_3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, build-ups of NaN_3 may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide build-up in plumbing.
8. Do not use Vial 9, Red Substrate Buffer if a precipitate is visible at room temperature.
9. Vial 11, Red Chromogen, contains Fast Red KL Salt and is labeled:
Toxic
R45 May cause cancer.
S35 This material and its container must be disposed of in a safe way.
S45 In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).
S53 Avoid exposure - obtain special instructions before use.
As a general rule, persons under 18 years of age are not allowed to work with this product. Users must be carefully instructed in the proper working procedure, the dangerous

properties of the product and the necessary safety instructions (per European Union Directive 94/33/EC).

Please refer to the Material Safety Data Sheet (MSDS) for additional information

10. Vial 12, Blue Chromogen, contains 5-amino-2-[3-[5-amino-1,3-dihydro-3,3-dimethyl-1-(4-sulfobutyl)-2H-indol-2-ylidene]-1-propenyl]-3,3-dimethyl-1-(4-sulfobutyl)-3H-Indolium, ter trifluoroacetate.

Caution: This product contains a substance, which has not yet been fully tested. As a general rule, persons under 18 years of age are not allowed to work with this product. Users must be carefully instructed in the proper working procedure, the dangerous properties of the product and the necessary safety instructions (per European Union Directive 94/33/EC).

Please refer to the Material Safety Data Sheet (MSDS) for additional information

11. Vial 13, Mounting Medium, contains Alkanes, C9-12, and is labeled:

Xn, Harmful

R65 Harmful: may cause lung damage if swallowed

S23 Do not breathe fumes

S24 Avoid contact with skin

S62 If swallowed, do not induce vomiting: seek medical advice immediately and show this container or label

Please refer to the Material Safety Data Sheet (MSDS) for additional information

12. 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 (14). 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.
13. Minimize microbial contamination of reagents to avoid erroneous results.
14. Incubation times and temperatures, or methods other than those specified, may give erroneous results.
15. Tissue fixation methods and thickness of specimen other than those specified may affect tissue morphology and/or signal intensity.
16. Avoid evaporation of *HER2/CEN-17* Probe Mix during hybridization by ensuring sufficient humidity in the hybridization chamber.
17. Reagents have been optimally diluted. Further dilution may result in loss of performance.
18. Unused solution should be disposed according to local, State, and Federal regulations.
19. Wear appropriate personal protective equipment to avoid contact with eyes and skin. Material Safety Data Sheet (MSDS) available on request.
20. It is recommended to follow standard procedure with respect to service and maintenance of the Dako Autostainer Instrument
21. People suffering from color vision deficiency should score samples with caution
22. As with any product derived from biological sources, proper handling procedures should be used.
23. Before preparation of the blue chromogen solution, the blue chromogen (vial 12) should be thoroughly mixed to ensure complete dissolution of the chromogen.
24. It is recommended to use two dropzones with 200 μ L in each (1 and 3) on the Dako Autostainer Instrument to cover the complete slide area and prevent drying of large tissue sections
25. If a specimen includes cluster of blue signals (amplification of CEN-17) that makes it difficult to count the red *HER2* signals and thereby interpret the staining, it is recommended to note the score and the staining pattern for reference. The user then

needs to refer to other test methods (e.g. FISH or IHC) to make a final conclusion on HER2 status.

26. Cases with increased number of blue CEN-17 signals (polysomy/aneusomy) resulting in a non-amplified result should be interpreted with caution. Please refer to guidelines.
27. Due to potential heterogeneity of the *HER2* signal distribution, it is important to perform a thorough scanning of the complete CISH stained specimen to evaluate signal distribution before selecting the area for signal enumeration.
28. The appearance of "Giant cells" within the tumor area should be noted and interpreted with caution since these cells can potentially affect the status of the specimen.

Storage

HER2 CISH pharmDx™ Kit is shipped as two separate items; SK109 Vial 2 (Pepsin) in a separate box and the remaining reagents in one kit box. SK109 Vial 2 (Pepsin) is shipped on dry ice and the kit box is shipped on cooling packs. **To ensure that Vial 2 (Pepsin) has not been exposed to high temperatures during transport dry ice should still be present in shipment upon receipt.**

Store all kit reagents at 2-8 °C, except Vial 13 (Tissue-Clear®-Based Mounting Medium). Vial 13 (Tissue-Clear®-Based Mounting Medium) should be stored at room temperature. Vial 3, 7 and 8 should be stored in the dark at 2-8 °C.

Pepsin and Red Chromogen (Vials 2 and 11) may be affected adversely if exposed to heat. Do not leave these components at room temperature.

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 (15).

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

Specimen Preparation

Specimens from biopsies, excisions or resections must be handled to preserve the tissue for CISH analysis. Standard methods of tissue processing for immunohistochemical staining should be used for all specimens (16).

Paraffin-embedded sections

Only tissue preserved in neutral buffered formalin and paraffin-embedded are suitable for use. Specimens should e.g. be blocked into a thickness of 3 or 4 mm and fixed for 18-24 hours in neutral buffered formalin. The tissues are then dehydrated in a graded series of ethanol and xylene, followed by infiltration by melted paraffin held at no more than 60 °C. Properly fixed and embedded tissues will keep indefinitely prior to sectioning and slide mounting if stored in a cool place (15-25 °C) (16-17). Other fixatives are not suitable.

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

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

A. Reagent Preparation

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

Day 1, Probe hybridization:

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 1

Dilute a sufficient quantity of Vial 5 (Wash Buffer 1 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.

Day 2, Immunohistochemical staining:

All reagents related to the immunohistochemical staining (Vial 6-13), except Red Chromogen (Vial 11), should be equilibrated to room temperature (20-25 °C) prior to reagent preparation. Reagents should be protected from light during equilibration.

A.5 Wash Buffer 2

Dilute a sufficient quantity of Wash Buffer 2 (Vial 6) (1:10) using distilled or deionised water for the staining procedure that is planned. Unused diluted solution may be stored at 2-8 °C for one month. Discard solution if cloudy in appearance. Wash Buffer 2 is interchangeable with Dako Wash Buffer, Code S3006.

A.6 Red Chromogen Solution

Before removing an aliquot of Red Chromogen from Vial 11, tip or mix the solution and centrifuge shortly. Prepare a sufficient quantity of Red Chromogen Solution but at least 1 mL. The proportion is 10 µL per 1 mL and the following procedure yields 1.01 mL of Red Chromogen Solution:

Transfer 1 mL of Red Substrate Buffer from Vial 9 to an empty, appropriate vial. Add 10 µL of Red Chromogen from Vial 11. Mix well.

Note: *The prepared Red Chromogen Solution should be used within 20 minutes. For best results, prepare the Red Chromogen Solution immediately before use.*

A.7 Blue Chromogen Solution

Before removing an aliquot of Blue Chromogen from Vial 12, thoroughly mix the chromogen and centrifuge briefly to remove liquid from the lid. Then immediately continue with the preparation of the blue chromogen solution.

Prepare a quantity of the blue chromogen solution that is sufficient for the entire run.

To prepare 1.075 mL blue chromogen solution; transfer 1 mL Blue Substrate Buffer from Vial 10 to an empty, appropriate vial. Add 75 μ L of the thoroughly mixed Blue Chromogen from Vial 12. Mix well.

If a smaller volume Blue Chromogen Solution is sufficient the scale can be reduced correspondingly – eg. for preparation of 430 μ L Blue Chromogen Solution, add 30 μ L thoroughly mixed Blue Chromogen from Vial 12 to 400 μ L Blue Substrate Buffer from Vial 10.

Note: It is recommended to prepare the blue chromogen solution *at least 30 minutes before use*. The Blue Chromogen Solution should be used within 8 days when stored at 2-8 °C in the dark.

A.8 Counterstain

Dilute a sufficient quantity of Dako Hematoxylin (Code S3301), 1:5 using distilled or deionised water for the staining procedure that is planned. Unused diluted hematoxylin may be stored at room temperature (20-25 °C) for one day.

B. Staining Procedure

B.1 Procedural notes

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

All reagents should be equilibrated to 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 (B3. Staining protocol, Step 1: Pre-Treatment, Method A). If microwave oven with sensing capability is used for pre-treatment (B3. Staining protocol, Step 1: Pre-Treatment, Method B) the diluted Pre-Treatment Solution should be equilibrated to room temperature **20-25 °C**.

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

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

Vial 4: The diluted Stringent Wash Buffer should be equilibrated to room temperature and **65 (\pm 2) °C** respectively prior to use.

Vial 5: The diluted Wash Buffer 1 should be equilibrated to room temperature **20-25 °C**.

Vial 6: Wash Buffer 2 (10x) should be equilibrated to room temperature **20-25 °C** prior dilution in room temperature equilibrated distilled or deionised water.

Vial 7: Peroxidase Block should be equilibrated to room temperature **20-25 °C**.

Vial 8: CISH Antibody Mix should be equilibrated to room temperature **20-25 °C**.

Vial 9: Red Substrate Buffer should be equilibrated to room temperature **20-25 °C**.

Vial 10: Blue Substrate Buffer should be equilibrated to room temperature **20-25 °C**.

Vial 11: Red Chromogen should be kept cold **at 2-8 °C** continuously.

Vial 12: Blue Chromogen should be equilibrated to room temperature **20-25 °C**.

Vial 13: Mounting Medium should be equilibrated to room temperature **20-25 °C**.

Coverslip sealant: Coverslip sealant may be applied at any temperature from **2-25 °C**

All steps must be performed at the outlined temperatures.

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 1 after the deparaffinization step for up to 1 hour at room temperature (20-25 °C) without affecting the results.

Immunohistochemical staining;

Dako Autostainer/Autostainer Plus instruments:

All incubations should be performed at room temperature (20-25 °C). The relevant volumes from Vial 7 (Peroxidase Block) and Vial 8 (CISH Antibody Mix) should be moved into Dako Autostainer Reagent Vials (Code S3425) and the remainder reagents (Red and Blue Chromogen Solutions, diluted Hematoxylin and Wash Buffer 2 for the 5 minutes post counterstain incubation) should be prepared directly in Autostainer Reagent Vials (Code S3425). Choose two dropzones (1 and 3) with 200 µL in each to prevent uneven staining and drying of slides.

Dako Autostainer Link instruments:

All incubations should be performed at room temperature (20-25 °C). Vial 7 (Peroxidase Block) and Vial 8 (CISH Antibody Mix) can be loaded directly on the instrument, while Red and Blue Chromogen Solutions and the Hematoxylin dilution should be prepared in User-Fillable Bottles. The reagents supplied with the kit are sufficient for 20 tests in 5 individual runs. Default setting is one dropzone with 200 µL reagent. Manually choose two drop zones (1 and 3) with 200 µL in each to prevent uneven staining and drying of slides.

When running 20 samples in one staining run, use 12 mL user-fillable bottles for preparation of Red and Blue Chromogen Solution.

Pay attention to incompatibility with other protocols in the same Autostainer run due to the stability of the Red Chromogen Solution after preparation. In order to accommodate the short stability of the Red Chromogen Solution, a maximum of 30 slides can be stained on the instrument in one staining run.

B.2 Deparaffinization

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 1 (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.

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

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. The regular use of in-house control slides is recommended for external quality control.

B.3 Staining protocol

DAY 1

Step 1: Pre-treatment

Pre-treatment can be performed either by use of water bath as described in the below Method A) or, alternatively, by use of microwave oven with sensing capability as described in the below 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 Pre-Treatment Solution in water bath. Heat the water bath and the Pre-Treatment Solution to 97-99 °C. Measure temperature inside jar with a calibrated thermometer to ensure correct temperature. Cover jars with lids in order to stabilize the temperature and avoid evaporation.

Immerse the room temperature deparaffinized sections into the preheated Pre-Treatment Solution in the staining jars. Re-check temperature and incubate for 10 (\pm 1) minutes at 95-99 °C. Remove the entire jar with slides from the water bath. Remove lid and allow the slides to cool in the Pre-Treatment Solution for 15 minutes at room temperature.

Transfer the slides to a jar with diluted Wash Buffer 1 (see INSTRUCTIONS FOR USE, Section A.3) for 3 minutes at room temperature (20-25 °C).

Replace Wash Buffer 1 and soak sections for another 3 minutes.

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

Fill a plastic jar with diluted room temperature (20-25 °C) 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 1 and soak for 3 minutes at room temperature (20-25 °C). Replace Wash Buffer 1 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 °C) while counting down the preset time (10 (\pm 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 °C) for at least 10 (\pm 1) minutes and manually stop the program after 10 (\pm 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

Pepsin incubation can be performed either at room temperature (20-25 °C) as described in the below Method A) or, alternatively, at 37 °C as described in the below Method B)

Tap off excess Wash Buffer 1. 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 μ L) of cold (2-8 °C) Pepsin (Vial 2) to cover specimen. Always store Pepsin at 2-8 °C.

Method A) Incubation at room temperature:

Incubate for 8 minutes at room temperature (20-25 °C). An incubation time of 8 minutes will be adequate for most specimens, but the optimal incubation time may depend on tissue fixation history and/or thickness of specimen and should be determined by the user.

Tap off Pepsin and soak sections in the diluted Wash Buffer 1 (see INSTRUCTIONS FOR USE, Section A.3) for 3 minutes at room temperature (20-25 °C).

Replace diluted Wash Buffer 1 and soak sections for another 3 minutes. Continue to dehydration.

Method B) Incubation at 37 °C:

Place specimen with Pepsin on a 37 °C heating block – e.g. Dako Hybridizer – and incubate for 2 minutes. An incubation time of 2 minutes will be adequate for most specimens, but the optimal incubation time may depend on tissue fixation and/or thickness of the specimen and should be determined by the user.

Tap off Pepsin and soak sections in diluted Wash Buffer 1 for 3 minutes at room temperature (20-25 °C).

Replace Wash Buffer 1 and soak sections for another 3 minutes. Continue to dehydration.

Dehydrate tissue sections through a graded series of ethanol (see INSTRUCTIONS FOR USE, Section A.4): 2 minutes in 70% ethanol, 2 minutes in 85% ethanol, and 2 minutes in 96% ethanol.

Allow tissue sections to air dry completely.

Step 3: HER2/CEN-17 Probe Mix, ready-to-use

The following step should be performed in a fume hood.

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

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

Prepare Dako Hybridizer* (Code S2450 or S2451) for a hybridization run. Start the Hybridizer and choose a program that will denature at 82 °C for 5 minutes and hybridize overnight (14-20 hours) at 45 °C (please refer to Dako Hybridizer Instruction Manual for details).

Place slides in the Hybridizer. Make sure that Humidity Control Strips (Code S2452) are saturated and optimal for use. Make sure the lid is properly closed and start program.

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

DAY 2

Step 4: Stringent Wash

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

Place 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 the water bath and the diluted Stringent Wash Buffer to 65 (±2) °C. Ensure that the temperature has stabilized. Cover jar with lid in order to stabilize the temperature and avoid evaporation. Measure the temperature inside the water bath jar with a calibrated thermometer to ensure correct temperature. Stringent Wash Buffer contains detergent and may become turbid at 65 °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 65 (±2) °C jar in the water bath. Perform stringent wash for exactly 10 minutes at 65 (±2) °C.

Remove slides from the diluted Stringent Wash Buffer, and soak sections in diluted Wash Buffer 1 for 3 minutes at room temperature (20-25 °C).

Change diluted Wash Buffer 1 and soak sections for another 3 minutes.

Step 5 Immunochemical staining protocol (Dako Autostainer instruments)

Step 5a: Select the HER2CISH protocol under pharmDx in the protocol selector. For first time users, the protocol should be installed prior to staining. On other Dako Autostainer platforms the protocol should be set up according to the program in figure 1. Alternatively contact Dako Technical Service for assistance in protocol installation for the automated platforms.

Step 5b: Load reagents

Step 5c: Load tissue slides. Do not allow tissue sections to dry while loading slides on the Dako Autostainer Instrument and during the staining procedure. Dried tissue sections may display increased background staining and weak signals.

Step 5d: Begin run. For Automated Link platforms choose “air blow” for completion

If the HER2CISH protocol is not present on your Autostainer Link platform, please contact Dako Technical Services.

Figure 1. Generic program for Dako Autostainer instruments

#	Category	Reagent	Volume	Incubation
1	Rinse	Buffer		0
2	Endogenous Enzyme Bloc	Peroxidase Block	200	5
3	Rinse	Buffer		0
4	Primary Antibody	CISH Antibody Mix	200	30
5	Rinse	Buffer		0
6	Rinse	Buffer		0
7	Substrate-Chromogen	Red Chromogen Solution	200	10
8	Rinse	Buffer		0
9	Rinse	Buffer		0
10	Substrate-Chromogen	Blue Chromogen Solution	200	10
11	Rinse	Buffer		0
12	Rinse	Buffer		0
13	Counterstain	Diluted Hematoxylin	200	5
14	Rinse	Buffer		0
15	Rinse	Buffer		5
16	Rinse	DI Water		0
17	Rinse	DI Water		0

Note: Choose two dropzones (1 and 3) with 200 µL in each.

Step 5e: Remove the slides from the Dako Autostainer instrument after the program has ended. Dry the sections for 30 minutes at 37 °C on Dako Hybridizer with the lid open. Allow slides to cool to room temperature before mounting.

Step 6: Mounting

Coverslip with Tissue-Clear®-Based Mounting Medium (Vial 13) and let the mounting medium harden at room temperature (20–25°C) for at least 30 minutes, preferably 60 minutes, prior to inspection.

Note: Subjecting specimens to alcohol or xylene prior to mounting must be avoided. It may compromise the sensitivity of the reaction and result in adverse effects on staining.

Use of Bright Field Microscope

Use a microscope objective of sufficient quality and magnification to allow for optimal scoring of specimens. Adjust light intensity to allow for easy separation of blue and red color. Focus up and down to find all of the signals in the individual nucleus.

Quality Control

1. Signals must be clear, well balanced in intensity, distinct and easy to evaluate.
2. Normal cells within the sample allow for an internal control of the staining run.
 - Normal cells should have 1-2 clearly visible blue signals indicating that the CEN-17 PNA Probe has successfully hybridized to the centromeric region of chromosome 17.
 - Normal cells should have 1-2 clearly visible red signals indicating that the *HER2* DNA Probe has successfully hybridized to the *HER2* amplicon.
 - In case of 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.
 - Numeric evaluation of normal cells should give a result corresponding to the expected value for normal diploid cells ($HER2/CEN-17=1$).
3. Nuclear morphology must be intact. 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 control slides.

Interpretation of Staining

Assessable tissue

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

Assessment of *HER2* CISH

Slide assessment:

Scan slide to account for possible heterogeneity between tumor areas. Make the assessment of the slide according to the guidelines below:

Signal enumeration: 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.

- Focus up and down to find all of the signals in the individual nucleus.

- If a signal appears to have more than one center of origin, and hence has a shape that differs significantly from a circular dot, it should be counted as two signals (please refer to signal counting guide below). A large signal that appears to have only one center of origin is counted as 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 blue signals, as clusters of *HER2* signals can cover the blue signals making them hard to see.
- In case of doubt, do not include the nuclei in the evaluation

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

Signal Counting Guide

1		Do not count. Nuclei are overlapping, not all areas of nuclei are visible.
2		Two blue signals, do not score nuclei with signals of only one color
3		Count as 3 blue and 12 red signals (cluster estimation)
4		Count as 2 blue and 1 red signal. Signals that appear to have more than one centre of origin, should be counted as two signals
5		Do not count (over- or under digested nuclei). Missing signals in the centre of nuclei
6		Count as 2 blue and 5 red signals. Signals that appear to have more than one centre of origin, should be counted as two signals
7		Count as 1 blue and 5 red signals
8		Count as 3 blue (1 blue out of focus) and 3 red signals
9		Cluster of red signals hiding blue signals. Go to a higher level of magnification to confirm presence or absence of blue signals. In case of doubt, do not count

Record counts

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 blue CEN-17 signals.

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

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

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

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

Limitations

1. *HER2* CISH pharmDx™ 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 CISH slide, and interpretation of the staining results.
2. CISH 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 should be used. Dako Hybridizer (Code S2450 or S2451) is recommended for denaturation and hybridization, however, if heating block and hybridization oven is used, they must be temperature-calibrated. 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. Excessive or incomplete counterstaining may compromise proper interpretation results.
6. The immunohistochemical staining procedure should be performed at ambient temperature of 20-25 °C.
7. Do not replace reagents with reagents carrying other lot numbers or with reagents from other manufactures.

Performance characteristics

Analytical sensitivity

The *HER2* DNA probes in *HER2/CEN-17* Probe Mix have been end-sequenced and mapped to confirm a total coverage of 218 kb including the *HER2* gene. The CEN-17 PNA probes in *HER2/CEN-17* Probe Mix have been tested individually and in combination to confirm their specific hybridization to the centromeric region of chromosome 17.

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

Furthermore, sensitivity was tested on 18 normal human breast epithelium specimens to verify that *HER2* CISH pharmDx™ Kit detects the target substances (*HER2* and CEN-17). The *HER2/CEN-17* ratios were between 0.97-1.09, i.e. in the interval expected of normal diploid cells.

Analytical specificity

Analytical specificity was tested to measure the assay's ability to solely identify target substances *HER2/CEN-17* without interference from other substances. Stained slides were evaluated for presence of signals in the absence of *HER2/CEN-17* Probe Mix or CISH Antibody Mix. No unspecific binding of the CISH Antibody Mix or the chromogens resulting in signals visible in either FISH or CISH were observed.

Robustness studies

The robustness of *HER2* CISH pharmDx™ Kit assay was tested by varying time and temperature for incubation with CISH Antibody Mix, Red Chromogen Solution, Blue Chromogen Solution and counterstaining. All other parameters of the procedure have been tested as part of the robustness studies for *HER2* FISH pharmDx™ Kit.

- Pre-treatment for 7, 10 and 13 minutes at 95-99 °C.
- Pre-treatment at 89, 92 and 95-99 °C for 10 minutes.
- Pepsin incubation times of 2, 5, 10, 15 and 18 minutes at room temperature.
- Denaturation temperatures of 72, 77, 82, 87 and 92 °C.
- Hybridization time of 17 hours at 40, 45 and 50 °C.
- Hybridization times of 10, 12 and 14 hours at 45 °C.
- The stringent wash was tested for 10 minutes at 60, 65 and 70 °C.
- Stringent wash was tested for 5, 10 and 15 minutes at 65 °C. Stringent wash for 10 minutes at 70 °C, and stringent wash for 15 minutes at 65 °C resulted in loss of signals, whereas no significant difference in results was observed at the other time/temperature combinations.
- Dilutions of Stringent Wash Buffer were tested: 1:10, 1:15, 1:20, 1:30 and 1:40. The 1:40 dilution of Stringent Wash Buffer resulted in loss of signals, whereas no significant difference in signal intensity was observed at the other dilutions.
- Antibody incubation time: 25, 27, 30, 33 and 35 minutes. 25 minutes incubation with antibody mix resulted in a loss of red signal intensity while no significant differences were observed at all other time points.
- Red chromogen solution incubation time for 8, 9, 10, 11 and 12 minutes
- Blue chromogen solution incubation time for 8, 9, 10, 11 and 12 minutes
- Counterstaining incubation time for 4, 5 and 6 minutes
- Counterstaining concentration at 1:4, 1:5 and 1:6 dilution

Note: For the robustness studies 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.

Repeatability

The repeatability of the *HER2*/CEN-17 ratio was investigated with *HER2* CISH pharmDx™ Kit using three consecutive sections from nine different breast cancer specimens. The coefficient of variance was found to be 1-7% with higher ratio resulting in higher CV. Repeatability on consecutive sections of breast cancer specimens with different thickness (3-7 µm) was tested with *HER2* CISH pharmDx™ Kit. The coefficient of variance of the *HER2*/CEN-17 ratio in this study was found to be 3-6%, i.e. in the same range as for tissue of equal thickness.

Reproducibility

The reproducibility of *HER2* CISH pharmDx™ Kit was tested for day-to-day and site-to-site reproducibility. Sections from 9 different breast cancer specimens were stained and analyzed on three days at three sites.

In the reproducibility study the analysis of breast cancer specimens using *HER2* CISH pharmDx™ Kit has shown reproducible results between days and between laboratories. According to a variance component model, measurements made on different sites and different days differed only slightly more than putative measurements made the same day at the same site (residual error) (Table 1).

Table 1. Variance component CV estimates

	Amplified	IHC 2+	Non-amplified
Site-to-site	15.8%	12.2%	9.0%
Day-to-day	13.6%	12.2%	8.4%
Residual error	13.1%	12.2%	8.4%

Observer-to-Observer study

Observer-to-observer variation was evaluated for slides stained with *HER2* CISH pharmDx™ Kit and read by three different observers. The 100 specimens in the study were represented by non-amplified (87%) and amplified (13%) and 61% being IHC *HER2* 2+.

Evaluation of the observer-to-observer variation resulted in an overall average CV at 12.4% for all specimens. This could be further divided into a CV at 10.9% for non-amplified and 22.6% for amplified specimens.

Table 2: Percent CV for non-amplified and amplified (the status of the individual specimen is determined by consensus agreement among the three observers).

	Number	Mean CV%
All cases	100	12.4
Consensus Non-amplified	87	10.9
Consensus Amplified	13	22.6

Comparative study

The clinical utility of *HER2* CISH pharmDx™ Kit was investigated in a clinical study comprising 365 invasive breast cancer specimens that were analyzed by *HER2* CISH pharmDx™ Kit and the PathVysion *HER-2* DNA Probe Kit (FISH). Specimens were consecutively collected and the specimen pool was enriched with 60 IHC 2+ specimens as determined by HercepTest™ and analyzed at a US reference laboratory. Test results from 350 specimens were eligible for statistical analysis, and the HercepTest™ IHC scores as well as their CISH *HER2* gene status are shown below in Table 3. A *HER2*/CEN-17 ratio below 2.0 indicates a non-amplified case and a *HER2*/CEN-17 ratio above or equal to 2.0 indicates an amplified case.

Table 3. Cross tabulation of HercepTest™ IHC scores and CISH *HER2* gene status

		HercepTest IHC score				Total
		0	1+	2+	3+	
CISH <i>HER2</i> gene status	non-amplified	98	108	95	5	306
	amplified	0	1	17	26	44
Total		98	109	112	31	350

The overall agreement between *HER2* status obtained by *HER2* CISH pharmDx™ Kit and PathVysion *HER-2* DNA Probe Kit (FISH) was 97.7% as shown in Table 4. The positive and negative agreement was 90.9% and 98.7%, respectively. McNemars test for a systematic bias between the two tests was not significant ($p=1.00$).

Table 4. Cross tabulation of CISH HER2 gene status versus PathVysion FISH HER2 gene status

		PathVysion FISH HER2 gene status		Total
		non-amplified	amplified	
CISH HER2 gene status	non-amplified	302	4	306
	amplified	4	40	44
Total		306	44	350

Overall agreement: $(342/350 \times 100) = 97.7\%$; (CI95 lower 95.7%; CI95 upper 98.9%)

Positive agreement: $(40/44 \times 100) = 90.9\%$; (CI95 lower 79.8%; CI95 upper 96.9%)

Negative agreement: $(302/306 \times 100) = 98.7\%$; (CI95 lower 96.9%; CI95 upper 99.6%)

Kappa: 0.90 (standard error 0.04)

In an exploratory analysis of the *HER2*/CEN-17 ratios obtained by the two methods the ratios have been plotted (Figure 1).

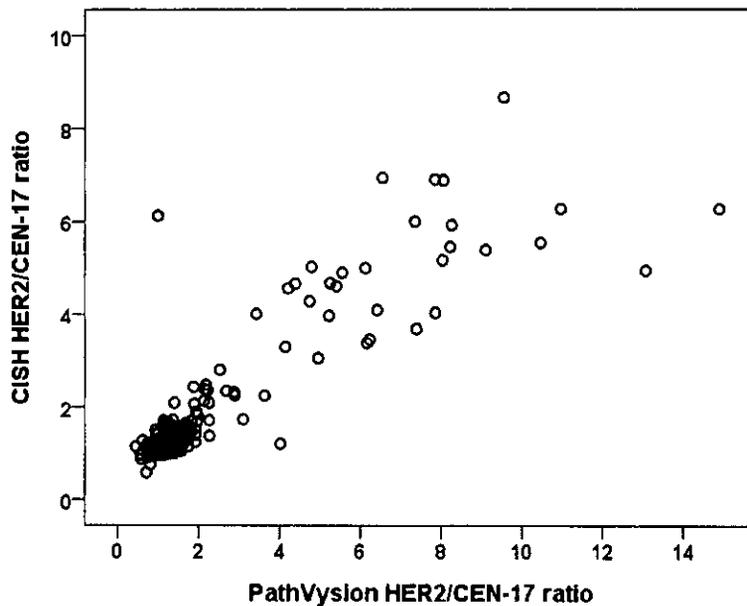


Figure 1. Plot of *HER2*/CEN-17 ratios for Dako *HER2* CISH pharmDx™ Kit and PathVysion *HER-2* DNA Probe Kit (FISH)

Linear regression of the logarithmically transformed *HER2*/CEN-17 ratios revealed a correlation coefficient at 0.90 and a slope of 0.71.

Slide evaluation time

The average slide evaluation time for *HER2* CISH pharmDx™ was 3:13 (min:sec) and for PathVysion *HER2* DNA Probe Kit (FISH) it was 4:02 (min:sec). In a paired, two tailed t-test the mean-difference in slide evaluation time was 0:49 (min:sec) which is significantly different from zero ($p < 0.001$).

CISH success rate

From a total of 364 CISH tests performed, 352 (96.7%) were successfully completed.

Prognostic value of *HER2* CISH pharmDx™ Kit

To link the prognostic value of *HER2* FISH pharmDx™ Kit to *HER2* CISH pharmDx™ Kit their concordance between test results was investigated. The prognostic value of *HER2* FISH

pharmDx™ Kit was previously shown based on the data in the DBCG (Danish Breast Cancer Cooperative Group) study 89D that analyzed *HER2* status using Dako *HER2* FISH pharmDx™ Kit. In the DBCG 89D study test results from 649 patient specimens were available for multivariate analysis, 417 had a *HER2*/CEN-17 ratio below 2.0 (normal or non-amplified *HER2* gene status) and 232 had a *HER2*/CEN-17 ratio above or equal to 2.0 (amplified *HER2* gene status).

From univariate analysis of the prognostic value of *HER2* amplification in node-positive patients (n=423) it was found that *HER2* gene amplification as determined by *HER2* FISH pharmDx™ Kit had a significant prognostic value for overall survival (Figure 2).

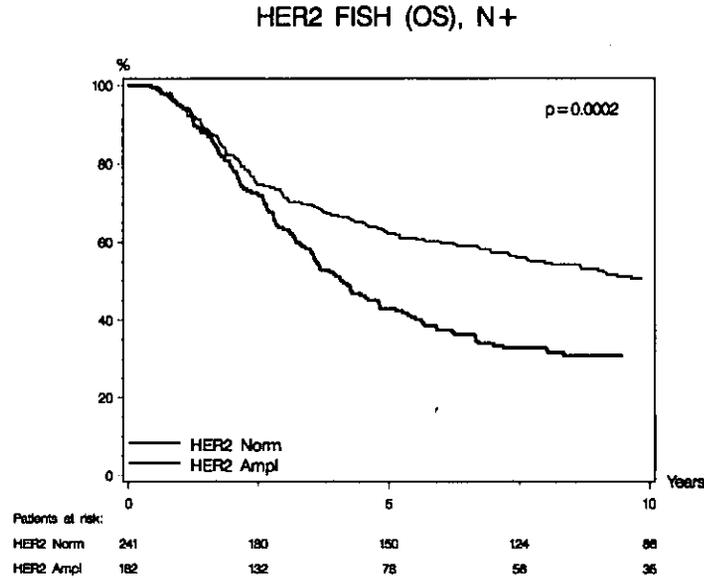


Figure 2. Kaplan Meier curve showing overall survival (OS) depending on *HER2* FISH amplification in node-positive patients (n=423).

Further multivariate analysis revealed that *HER2* FISH amplification had an independent prognostic value in node-positive patients with a hazard ratio corresponding to *HER2* amplification for overall survival at 1.40 (hazard ratio CI95 limits: 1.07-1.85), p=0.015.

The concordance between *HER2* FISH pharmDx™ Kit and *HER2* CISH pharmDx™ Kit assays was investigated in a clinical study comprising 365 invasive breast cancer specimens. Test results from 348 specimens were eligible for statistical analysis of *HER2* status, and the cross tabulation of *HER2* status obtained by the two assays are presented below in Table 4. The concordance data show an overall agreement between Dako FISH and CISH pharmDx™ Kit assays of 98.3%, with positive and negative agreements at 93.2% and 99.0%, respectively. Therefore, the data shows that the prognostic value of the *HER2* FISH pharmDx™ Kit analysis result can be acquired using the *HER2* CISH pharmDx™ Kit assay.

Table 5. Cross tabulation of *HER2* gene status obtained by *HER2* CISH pharmDx™ Kit and *HER2* FISH pharmDx™ Kit.

		Dako FISH <i>HER2</i> status		Total
		non-amplified	amplified	
Dako CISH <i>HER2</i> status	non-amplified	301	3	304
	amplified	3	41	44
Total		304	44	348

Overall agreement: $(342/348 \times 100) = 98.3\%$ (CI95: 96.5%; 99.3%)

Positive agreement: $(41/44 \times 100) = 93.2\%$ (CI95: 82.9%; 98.0%)

Negative agreement: $(301/304 \times 100) = 99.0\%$ (CI95: 97.4%; 99.7%)

Kappa: 0.92 (SE: 0.03)

Trouble shooting

Problem	Probable cause	Suggested Action
<p>1. No signals or weak signals</p>	<p>1a. Kit has been exposed to high temperatures during transport or storage</p> <p>1b. Pre-treatment conditions incorrect</p> <p>1c. Evaporation of Probe Mix during hybridization</p> <p>1d. Microscope settings not optimal</p> <ul style="list-style-type: none"> - Inadequate light - Higher magnification (40x or 60x objectives) <p>1e. Red and/or Blue Chromogen Solution are not used within the indicated time</p> <p>1f. Automated staining</p> <ul style="list-style-type: none"> - Reagents are not used in proper order - Insufficient reagent volume - Inadequate incubation times - Wrong dropzone <p>1g. Drying of slides</p>	<p>1a. Check storage conditions. Ensure that dry ice was present when Vial 2 (Pepsin) was received. Ensure that the cool packs were cold when the kit was received. Ensure that the complete kit is stored at maximum 2-8 °C in the dark. Mounting medium (Vial 13) should be stored at room temperature.</p> <p>1b. Ensure that the recommended pre-treatment temperature and time are used.</p> <p>1c. Ensure sufficient humidity in the hybridization chamber</p> <p>1d. Try different microscope settings. In case of doubt, contact your local microscope vendor</p> <p>1e. Prepared Red Chromogen Solution must be used within 20 minutes. Blue Chromogen Solution should be prepared 30 minutes before use and must be used within 8 days (when stored at 2-8 °C in the dark).</p> <p>1f. Review Programming Grid, slide layout and Reagent Map prior to commencing the run.</p> <p>1g. Do not allow the tissue sections to dry at any time in the staining</p>

(placeholder)

		procedure
2. No blue signals	<p>2a. Stringent wash conditions incorrect</p> <p>2b. Wrong dilution of Blue Chromogen</p> <p>2c. Problems with preparation of Blue Chromogen Solution</p>	<p>2a. Ensure that the recommended stringent wash temperature and time are used, and that coverslips are removed before performing stringent wash</p> <p>2b. Prepare Blue Chromogen Solution by mixing 75 μL Blue Chromogen (vial 12) in 1 mL Blue Substrate Buffer (vial 10)</p> <p>2c. Blue Chromogen (vial 12) was not mixed before preparation of Blue Chromogen Solution</p>
3. No red signals	<p>3a. Pre-treatment conditions incorrect</p> <p>3b. Incorrect use of Red Chromogen Solution</p> <p>3c. Wrong dilution of Red Chromogen</p>	<p>3a. Ensure that the recommended pre-treatment temperature and time are used</p> <p>3b. Prepared Red Chromogen Solution should be used within 20 minutes (including incubation time)</p> <p>3c. Prepare Red Chromogen Solution by mixing 10 μL Red Chromogen (vial 11) in 1 mL Red Substrate Buffer (vial 9)</p>
4. Areas without signal	<p>4a. Probe volume too small</p> <p>4b. Air bubbles caught during Probe Mix application or mounting</p> <p>4c. Reagent volume too small</p> <p>4d. Air bubbles caught during mounting</p> <p>4e. Drop zone</p>	<p>4a. Ensure that the probe volume is large enough to cover the area under the coverslip</p> <p>4b. Avoid air bubbles. If observed, gently tap them away using forceps</p> <p>4c. Ensure that the reagent volume is large enough to cover the tissue area</p> <p>4d. Avoid air bubbles. Use the mounting medium supplied with the kit.</p> <p>4e. Make sure dropzone is aligned with location of tissue section. Use two dropzones (1 and 3)</p>
5. Excessive background	5a. Inappropriate tissue	5a. Ensure that only

<p>staining</p>	<p>fixation</p> <p>5b. Paraffin incompletely removed</p> <p>5c. Stringent wash temperature too low</p> <p>5d. Evaporation of Probe Mix during hybridization</p> <p>5e. Slides not thoroughly rinsed</p> <p>5f. Sections dried while loading the Autostainer</p> <p>5g. Sections dried during automated staining procedure</p>	<p>formalin-fixed, paraffin-embedded tissue sections are used</p> <p>5b. Follow the deparaffinization and rehydration procedures outlined in Section B.2</p> <p>5c. Ensure that the stringent wash temperature is 65 (± 2) °C</p> <p>5d. Ensure sufficient humidity in the hybridization chamber. Use humidity control strips</p> <p>5e. Use fresh wash buffer. Ensure that the Autostainer is properly primed prior to running. Check to make sure that adequate buffer is provided for entire run</p> <p>5f. Ensure sections remain wet with buffer while loading and prior to initiating run</p> <p>5g. Verify that the appropriate volume of reagent is applied to slides. Make sure that the Autostainer is run with fume hood in the closed position and is not exposed to excessive heat.</p>
<p>6. Poor tissue morphology</p>	<p>6a. Incorrect Pepsin treatment</p> <p>6b. Overdigested tissue</p>	<p>6a. Adhere to recommended Pepsin incubation times. See section B.3, step 2. Ensure that Pepsin is handled at the correct temperature. See Section B.1</p> <p>6b. Shorten the pepsin incubation time in the Dako FISH staining procedure. For neutral buffered formalin (24 hours) and paraffin-embedded tissue 2 minutes pepsin incubation at 37 °C is a good starting point.</p> <p>There is a balance between tissue morphology and signal</p>

	<p>6b. Incorrect pre-treatment conditions may result in unclear or cloudy appearance</p> <p>6c. Too long Pepsin treatment or very thin section thickness may cause ghost cells or donut cells to appear.</p>	<p>intensity/quality that the user should be aware of when making a pepsin gradient in the FISH procedure as both under- and overdigestion of tissue specimens has an effect on signal intensity/quality.</p> <p>6b. Ensure that the recommended pre-treatment temperature and time are used</p> <p>6c. Shorten Pepsin incubation time. See section B.3, step 2. Ensure that the section thickness is 4-6 μm.</p>
<p>7. The hematoxylin counterstaining makes it difficult to see the specific <i>HER2</i> and CEN-17 signals</p>	<p>7a. Hematoxylin counterstaining too strong</p>	<p>7a. Further dilution of hematoxylin or shorter incubation time needed</p>
<p>8. Excessive strong specific staining</p>	<p>8a. Reagent incubation time too long</p> <p>8b. Inappropriate wash solution used</p>	<p>8a. Review staining procedure instructions</p> <p>8b. Use only Wash Buffer recommended with the kit (Wash buffer 2, supplied with the kit or Dako Wash Buffer, Code S3006)</p>

Appendix 1

HER2 CISH pharmDx™ Kit, Code SK109

Protocol Checklist

Staining Run Log ID: _____

Date (day 1) of the run: _____

HER2 CISH pharmDx™ Kit, SK109 Lot: _____

Specimen ID(s): _____

Equipment ID(s): _____

Tissue fixed in neutral buffered formalin	Yes	No
---	-----	----

Date of dilution/expiration of the 1 x Wash Buffer 1 (Vial 5 diluted 1:20)	/
Date of dilution/expiration of the 1 x Wash Buffer 2 (Vial 6 diluted 1:10)	/

DAY 1

Step 1: Pre-treatment	
Date of dilution/expiration of Pre-Treatment Solution (Vial 1 diluted 1:20)	/
Measured temperature of Pre-Treatment Solution (95-99 °C) if water bath (method A) is used for heating If microwave oven (method B) is used, mark with a dash	° C
Pre-treatment (10 minutes), and cooling (15 minutes)	
Wash in Wash Buffer 1 (Vial 5 diluted 1:20) (2 x 3 minutes)	
Step 2: Pepsin	
Duration of Pepsin (Vial 2) treatment at 37 °C or	Minutes
Duration of Pepsin (Vial 2) treatment at room temperature	Minutes
Wash in Wash Buffer 1 (Vial 5 diluted 1:20) (2 x 3 minutes)	
Dehydrate slides (3 x 2 minutes) in graded series of ethanol and air dry	
Step 3: HER2/CEN-17 Probe Mix	
Apply Probe Mix (Vial 3), coverslip and seal with Coverslip Sealant	
Measured denaturation temperature (82 °C) if equipment other than Dako Hybridizer is used	° C
Denaturation for 5 minutes	
Measured hybridization temperature (45 °C) if equipment other than Dako Hybridizer is used	° C
Hybridization overnight (protect from light)	
	Hours

DAY 2

Step 4: Stringent Wash	
Date of dilution/expiration of the Stringent Wash Buffer (Vial 4 diluted 1:20)	/
Measured temperature of Stringent Wash Buffer (65 °C)	° C
Stringent wash (10 minutes) after removing the coverslip	
Wash in Wash Buffer 1 (Vial 5 diluted 1:20) (2 x 3 minutes)	

(placeholder)

Step 5: Immunohistochemical staining (Dako Autostainer Instrument)	
Step 6: Mounting:	
Dry for 30 minutes at 37 ° C, cool to room temperature, then	
Apply Mounting medium (Vial 13) and coverslip	
Wait at least 30-60 minutes before inspection	

Comments: _____

Date and signature, Technician: _____

Appendix 2

HER2 CISH pharmDx™ Kit, Code SK109

Scoring Scheme

Staining Run Log ID: _____

Date (day 1) of the run: _____

HER2 CISH pharmDx™ Kit, SK109 Lot: _____ Specimen ID(s): _____

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

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

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

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

- Ratio < 2: *HER2* gene amplification was not observed
- Ratio ≥ 2: *HER2* gene amplification was observed

Date and signature, Technician: _____

Date and signature, Pathologist: _____

For scoring guidelines: See Interpretation of Staining

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 REF	Catalogue number	 Store in the dark	 Toxic	 Manufacturer
 IVD	In vitro diagnostic medical device	 Contains sufficient for <n> tests	 Harmful	
 Consult instructions for use	 LOT	Batch code	 Extremely flammable	
 Temperature limitation	 Use by		 Dangerous for the environment	

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