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For Professional Use Only

Human HER2 gene amplification detection kit Instructions Manual

CE IVD

[Product Name] Human HER2 gene amplification detection kit

[Package Specifications] 10 Tests/box

[Intended Usage]

This kit uses fluorescence in situ hybridization to detect the HER2 gene amplification status in-vitro. The test sample is a paraffin-embedded specimen of breast cancer tissue. The product has not been clinically validated in conjunction with HER2 targeted therapies, and its clinical testing capabilities have been confirmed by comparative trial studies with companion diagnostics that have targeted drug validation. The test results of the product should not be used as the sole basis for individualized treatment. The clinician should make comprehensive judgment on the test results in combination with the patient's condition, drug indications, treatment response, and other laboratory testing indicators.

Human epidermal growth factor receptor 2 (HER2, also known as Neu, ErbB-2, CD340, or p185) is a proto-oncogene HER2/neu located at the 17q12 of the human chromosome 17 long arm. Encoded, it is one of epidermal growth factor receptor (EGFR/ErbB) family members, has tyrosine kinase activity and is involved in signal transduction of cell growth and differentiation. The carcinogenic mechanism of HER2 oncogene includes inhibition of cell apoptosis, promotion of cell proliferation, increase of tumor cell invasiveness, and promotion of tumor angiogenesis and lymphangiogenesis.

Breast cancer is one of the most serious malignant tumors that threaten women's health. Its incidence has been increasing year by year. HER2 gene amplification is found in 20% to 30% of diagnosed breast cancer patients. Patients with HER2-positive breast cancer often show high levels of tumor malignancy, poor treatment outcomes, and poor prognosis, but can benefit from specific HER2-targeted drug therapies. Therefore, the detection of HER2 gene amplification status in breast cancer patients is the precondition for the screening of HER2-targeted breast cancer patients and the curative effect prediction.

[Detection Principle]

The kit is based on fluorescence in situ hybridization technology. A nucleic acid probe is labeled with fluorescein. The target gene is detected with homologous complementary to the nucleic acid probe used. Both after denaturation, annealing and renaturation, the hybrid of the target gene and the nucleic acid probe can be formed, and the qualitative, quantitative or relative positioning analysis of the gene to be measured under the microscope can be performed by the fluorescence detection system.

This kit uses the rhodamine fluorescein (RHO)-labeled orange probe (HER2 probe) to detect the HER2 gene, and the fluorescein isothiocyanate (FITC)-labeled green probe (CEP17 probe) to detect chromosome 17 centromere sequence can be used to bind two probes to the target detection area by in situ hybridization. The number of signals corresponding to the CEP17 probe reflects the number of chromosomes at the target area, and the number of signals from the HER2 probe reflects the copy number of the HER2 gene at the target site. By the ratio of the number of HER2 probes and the number of CEP17 probe signals, the amplified state of the HER2 gene in the tissue to be detected can be determined. **[Product Main Components]**

The kit consists of HER2 orange probe and CEP17 green probe, as shown in Table 1. The reagents not provided in the kit are shown in Table 2.

Table 1 Kit composition

Component name	Specifications	Quantity	Main components
HER2/CEP17 dual color probe	100μl/Tube	1	50%deionizedformamide,2xSSC,10%dextransulfate,HER2orange probe, CEP17 green probe

Table 2 List of reagents not provided

Reagent name	Purity	Reagent name	Purity
Sodium chloride	Analytical purity AR	NP-40	Analytical purity AR
Sodium citrate	Analytical purity AR	Xylene	Analytical purity AR
Anhydrous ethanol	Analytical purity AR	Protease K	≥40 units/g

[Storage conditions & Validity]

Keep sealed away from light at -20℃±5℃. The product is valid for 12 months. Avoid unnecessary repeated freezing and thawing that should not exceed 10 times. After opening, within 24 hours for short-term preservation, keep sealed at 2~8℃ in dark. For long-term preservation after opening, keep the lid sealed at -20℃±5℃ away from light. See the label of the kit for the production date and expiration date.

[Applicable Instruments]

1. Fluorescence microscopy imaging system includes fluorescence microscope and filter sets. The kit is labeled with orange fluorescein, and the filter set compatible with the fluorescent labeled dye should be selected.

Orange fluorescence: The maximum excitation wavelength is 547nm and the maximum emission wavelength is 565nm.

Green fluorescence: The maximum excitation wavelength is 495nm and the maximum emission wavelength is 517nm.

Fluorescence microscopy imaging system should use a microscope with orange and green channels.

2. Automatic hybridization instrument: Strict temperature uniformity is required, and the temperature difference should be ≤1℃.

[Sample Requirements]

1. Applicable specimen types: Paraffin-embedded specimens from surgical resection of breast cancer.

2. The tissue should be fixed with 4% neutral formaldehyde solution within 1 hour after isolation. After tissue fixation, it is routinely dehydrated and embedded in paraffin.

3. Paraffin section thickness could affect the experimental results. The recommended slice thickness is 4~5μm.

4. Paraffin-embedded tissue samples from breast cancer should be selected from representative tumor tissue wax blocks and confirmed by HE staining.

5. It is recommended to select paraffin-embedded tissue specimens within 5 years preservation period.

[Testing Method]

1. Related reagents

The following reagents are required for the experiment but not provided in this kit

① 20×SSC (sodium citrate buffer), pH 5.3±0.2

Sodiumchloride	176g
Sodiumcitrate	88g

Weigh 176g of sodium chloride and 88g of sodium citrate, dissolve in 800mL of deionized water, adjust the pH to 5.3±0.2 at room temperature, and complete to 1 L with deionized water. High-pressure steam sterilization, stored at 2~8℃, the solution shelf life is of 6 months. Discard if the reagent appears cloudy (turbid) or contaminated.

② 2×SSC, pH 7.0±0.2

Take 100mL of the above 20xSSC, dilute with 800mL deionized water, mix, adjust the pH to 7.0±0.2 at room temperature, complete to 1L with deionized water, stored at 2~8℃, the shelf life is of 6 months. Discard if the reagent appears cloudy (turbid) or contaminated.

③ Ethanol Solution: 70% ethanol, 85% ethanol

Dilute 700ml, 850ml of ethanol with deionized water to 1L. The shelf life is of 6 months. Discard if the reagent appears cloudy (turbid) or contaminated.

④ Protease K

Protease K stock solution (20mg/mL): Weigh 0.1g of proteinase K dry powder, dissolve in 5mL 2xSSC (pH value 7.0). Gently mix the solution till completely dissolved, and store at -20°C. Shelf life is 6 months.

Protease K working solution (200μg/mL): Dissolve 0.8mL Proteinase K stock solution in 80mL 2×SSC (pH value 7.0), mix well and the solution is ready for immediate use.

⑤ 0.3% NP-40/0.4xSSC solution, pH 7.0 ~ 7.5

NP-40	0.6mL
20xSSC	4mL

Take 0.6mL NP-40 and 4mL 20×SSC, add 150mL deionized water, mix, adjust the pH to 7.0 ~ 7.5 at room temperature, with deionized water complete to a volume of 200mL. Stored at 2~8℃, the shelf life is of 6 months. Discard if the reagent appears cloudy (turbid) or contaminated.

⑥0.1% NP-40/2×SSC solution, pH 7.0 ± 0.2

NP-40	0.2mL
20xSSC	20mL

Take 0.2mL NP-40 and 20mL 20×SSC, add 150mL deionized water, mix, adjust the pH 7.0±0.2 at room temperature, with deionized water complete to a volume of 200mL. Stored at 2~8oC, the shelf life is of 6 months. Discard if the reagent appears cloudy (turbid) or contaminated.

⑦ Diamidinyl phenylindole (DAPI) counterstain

Use commercially available anti-quenching DAPI counterstain.

⑧ Xylene

2. Pretreatment

It is recommended to select specimens with known positive and negative HER2 gene amplification as controls.

① Sectioning: Place on clean slides tissue section fixed in neutral formalin and paraffin-embedded.

② Heating slices: Place the slices in the heating machine and heat overnight at 65°C.

③Dewaxing: Immerse the tissue sections in xylene for 10 minutes, dewax, repeat once, and then immediately immerse in 100% ethanol for 5 minutes.

④ Rehydration: At room temperature, place in 100% ethanol, 85% ethanol, and 70% ethanol for 2 minutes each the tissue sections, immediately immerse in deionized water for 3 minutes. Take out the slices and use a lint-free tissue to absorb excess water around the tissue.

⑤ Water treatment: Soak tissue sections in deionized water at 95°C for 30 to 40 minutes (deionized water is applied in a water bath to preheat).

⑥ Washing: At room temperature, soak the tissue sections in 2xSSC solution and rinse twice for 5 minutes each.

⑦ Proteinase K treatment: Soak the tissue sections in proteinase K working solution and treat at 37°C for 5-30 minutes.

⑧ Washing: At room temperature, soak the tissue sections in 2xSSC solution and rinse twice for 5 minutes each.

⑨ Dehydration: Placed the tissue sections in 70% ethanol, 85% ethanol and 100% ethanol for 2 minutes each, and then dry naturally.

3. Denaturation and Hybridization

The following operations should be performed in a darkroom.

① Take the dual-color probe. Mix and centrifuge briefly. Take 10μl droplet in the cell and drop in the hybridization zone, immediately cover 22mmx22mm glass slide area; spread evenly without bubbles the probe under the glass slide covered area and seal edges with rubber (edge sealing must be thorough to prevent dry film from affecting the test results during hybridization).

② Place the glass slides in the hybridization instrument, denature at 83°C for 5 minutes (the hybridizer should be preheated to 83°C) and hybridize at 42°C for 2 to 16 hours.

4. Washing

The following operations should be performed in a darkroom.

① Take out the hybridized glass slides, remove the rubber on the coverslip and immediately place the slides in a 0.3% NP-40/0.4x SSC solution at 67°C. Shake for 1-3 seconds, remove the coverslip and continue to soak the glass slides for 1-2 minutes.

② At room temperature, place the slices in 0.1%NP-40/2xSSC solution, oscillate for 1-3 seconds and soak for 1-2 minutes.

③ At room temperature, place the slices in 70% ethanol, soak for 1-3 minutes, and naturally dry in the dark.

5.Counterstaining

The following operations should be performed in a darkroom.

Dip 10~15μL of DAPI counterstain into the hybridization area of the glass slide, immediately cover and place in dark for 10~20 minutes, and then use the suitable filter to observe the sections under the fluorescence microscope.

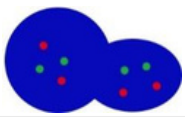
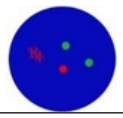
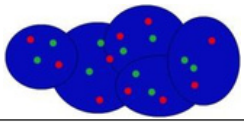
6. FISH results observation

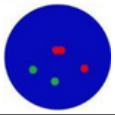
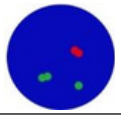
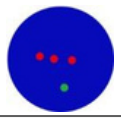
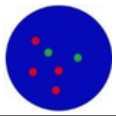
Place the stained sections under a fluorescence microscope and the area of the breast cancer cells is first confirmed under a low magnification objective (10×); under magnification objective (40×) a uniform cells distribution is observed; then the nucleus size uniformity, nuclear boundary integrity, DAPI staining uniformity, no nuclei overlapping, cells clear signal are observed in the high magnification objective (60x, 100x). Select randomly 20 tumor cells at least and count the orange and green signals in the nuclei.

[Positive Value Reading]

1. Signal count and ratio measurement

Table 3 Dual-color for signal counting guide

● HER-2, orange signal ● CEP 17, green signal	
	Nuclei are partially overlapping but signals are not in overlapping area. Count as 2 orange signals and 2 green signals in each nucleus.
	Count as 2 orange signals and 2 green signals. One orange signal is diffuse.
	Do not count. Nuclei are overlapping, nuclei areas are not all visible and some signals are in overlapping area.

	Count as 2 orange signals and 2 green signals.
	Count as 1 orange signal and 2 green signals.
	Count as 2 orange signals and 1 green signal.
	Count as 3 orange signals and 1 green signal.
	Count as 4 orange signals and 2 green signals.

The number of HER2 signals and the number of signals of CEP17 in 20 tumor cells were observed and recorded (Table 3). The total number of HER2 signals indicates the total HER2 copy number, and the total number of CEP17 signals indicates the total number of chromosomes 17 in the target area. Calculate the ratio value (Ratio = Total number of HER2 signals / Total number of CEP17 signals).

2. Breast cancer FISH result determination

① When the ratio is ≥2.0, the tissue is considered positive for HER2 gene amplification.

② The ratio is <2.0, but HER2 gene amplification is also positive when HER2 gene copy number/cell number ≥6.0.

③ If the HER2 orange signal is clustered or the ratio is greater than 20, the HER2 gene is directly considered to be amplified.

Negative HER2 gene amplification

Ratio <2.0, and HER2 gene copy number/cell number<4.0 is negative for HER2 gene amplification.

The HER2 gene amplification status is uncertain

When the ratio<2.0, 4.0 ≤HER2 gene copy number/cell number <6.0, the amplification result of HER2 gene is uncertain. For cases with uncertain results, it is necessary to count the signals in 20 nuclei, sum up the two count results or count again the results by another analyst. If results still in critical value range, immunohistochemistry detection should performed (if FISH is not done anteriorly). Different tissue blocks can be selected for re-testing.

[Results Interpretation]

1. The CEP17 probe is used as an internal control in this kit. The hybridization is regarded as successful when more than 75% of the nuclei in the tissue show a dual-color signal. The dual-color signals are used as mutual controls to compare the cancerous and non-cancerous cells.

2. The CEP17 probe is added to the fluorescence in situ hybridization dual-probe assay to detect the chromosome 17 at the same time detect the HER2 gene, thereby differentiate between chromosome 17 aneuploidy and HER2 gene amplification, especially at low levels of amplification. The mean signal number of the centromere of chromosome 17 in a tumor cell is ≥3, and it is a chromosome 17 polymorphism. Studies have shown that the whole chromosome 17 polymorphism is rare, and the polymorphism of the chromosome 17 centromere does not represent the entire chromosome 17 polymorphism. There is a co-amplification of the HER2 gene and the centromere on chromosome 17 in some breast cancers. More and more scholars believe that HER2 copy number is more important for the judgment of HER2 gene amplification than HER2/CEP17 ratio. Therefore, in addition to the HER2/CEP17 ratio reported in the HER2 gene in situ hybridization assay, the HER2 gene copy number and the chromosome 17 centromere should also be reported separately.

3. FISH test failure should be considered when the following conditions occur, including

① No positive or negative results were expected.

② Tumor lesions are too small, with difficulty to observe two tumor regions and count.

③ Countable signal cells < 75%.

④ Over 10% of the fluorescence signal is located outside the nucleus.

⑤ Difficulty to distinguish the nuclei structure.

⑥Verystrongauto-fluorescence.

Table4FAQandSolutions

Question	Possible Cause	Recommended Solution
Too strong	Slides were not cleaned properly	Slides washing with anhydrous ethanol.

background	before specimen's preparation.	
	Incomplete washing after hybridization.	Ensure that the washing solution is prepared according to instructions; make sure that the washing solution pH and temperature are correct; remove the coverslip and repeat the washing.
	Filter sets improper use	Replace the appropriate filter set to weaken the background light.
	Improper hybridization conditions.	Ensure that the hybridization instrument temperature is 42°C
	Low washing temperature.	Ensure that the solution temperature of the washing glass slides is up to the required temperature.
	Washing solution strength is too low.	Ensure that the washing solution is prepared according to instructions. (Low SSC concentration and high NP-40 concentration are beneficial to increase the washing solution strength).
The dye is too weak	Dystaining	Remove coverslips and soak for 5 minutes in a 2xSSC/0.1% NP-40 washing solution at room temperature. Place the slides sequentially in 70%, 85%, and 100% ethanol solutions for 1 minute each for gradient dehydration and then re-dye.
	Obsolete dye agent or Excessive illumination	Ensure that the dye agent is stored at -20°C to avoid light, and ensure that the dye agent is not invalid.
No signal or weak signal	Sample incomplete denaturation	Ensure that the hybridization instrument temperature is 83°C, preheat it for at least 10 minutes in advance.
	Incomplete mix before use of the probe and hybridization buffer	Blow the probe mixture and mix the probe thoroughly. Centrifuge briefly.
No signal or weak signal	The probe mixture dries too fast on the glass slide	The target area should be immediately covered after the probe mixture is dropped with cover glass; when washing, only one cover glass on the slide can be removed at a time and the slide can be immersed in the washing solution immediately before the next one is removed.
	Bubble formation under cover glass during hybridization.	Cover the surface of the probe mixture and gently squeeze to release the bubbles.
	Inappropriate hybridization conditions	Ensure that specified hybridization time and temperature are observed; that no gaps are left in the rubber seal, and that the time of hybridization is adjusted.
	Incorrect washing or inappropriate washing conditions	Ensure that the washing solution is prepared according to the product specification; ensure that the temperature of the washing solution reaches the specified temperature for the washing step; ensure that the thermometer and pH meter are correctly calibrated; remove the cover glass before the slide is immersed in the washing solution.
No signal or weak signal	Inappropriate probe storage or specimen slides	Ensure that probes are stored in the dark at -20°C. Place the unhybridized slides dry at -20°C for a long storage period of time or at room temperature for a short storage period of time. After hybridization, store in dark the (hybridized) slides at -20°C. The storage period should not exceed 6 months.
	Dye agent incorrect usage Dye agent too high brightness	Remove the coverslip and soak the slides in 2xSSC/0.1% NP-40 solution for 5 minutes at room temperature. Place slides sequentially in 70%, 85% and 100% ethanol solutions for 1 minute each to dehydrate. Dry the slides naturally and add the dye agent
	Inappropriate filter set selection for observation	Use the appropriate filter set to observe the fluorescence of the probe.

For details and more support, please contact Wuhan HealthCare Biotechnology Co., Ltd. Technical Department at bd@healthcare-bio.com

[Test Method Limitations]

1. This kit is an in vitro diagnostic reagent. The clinical judgment of the test results should be combined with the patient's medical history and other clinical diagnostic results to conduct a comprehensive assessment, and should not be used as the sole basis for clinical diagnosis and treatment.
2. The test results are affected by the sample source, sample collection process, sample quality, sample transport conditions, and sample pretreatment. At the same time, the subjectivity of the result assessment may lead to false positive or false negative test results. Users should understand the potential errors and accuracy limitations in the detection process.
3. The test results, if inconsistent with histopathological features, should be verified by pathological diagnosis or reexamination.
4. The performance index of this product is based on the testing procedures described in the manual. Any changes may lead to test results alteration.
5. This reagent has been validated only on tissues that have been fixed with 4% of neutral formaldehyde fixed paraffin embedded tissues and may not be used for other sample types or flow cytometry and other purposes.

[Product Performance Index]

1. The kit packaging should be intact and not damaged. The labels should be complete and clear; each liquid reagent should be marked clearly and without leakage.
2. After the probe effective hybridization with peripheral blood lymphocytes, it should emit a fluorescent signal that can be identified by the naked eye under a fluorescence microscope.
3. Paraffin-embedded tissue sections from 4 breast cancer patients with negative HER2 gene amplification were analyzed for fluorescent signals and the results were negative.
4. Paraffin sections from four cases of breast cancer patients positive for HER2 gene amplification were examined and analyzed for fluorescent signals. Results were all positive.
5. Sensitive detection reference (product) analyzed 100 sets of chromosome 17 of 50 cells in the metaphase division. At least 98 sets of

chromosome 17 showed a green fluorescent signal and 1 orange-red fluorescent signal.

6. Specific detection reference (product) analyzed 100 chromosomes 17 of 50 cells in the metaphase division, and at least 98 chromosomes 17 display 1 specific green fluorescence signal in the centromere region, 1 specific orange fluorescent signal in the target region.

7. The clinical study of this kit was designed using a synchronous blind test method, and a companion diagnostic reagent that has been targeted for drug validation (Abbott Molecular's "HER-2 Gene Detection Kit for Breast Cancer (FISH Method)" (CFDA Registration (2013) No. 3402619) was used as a comparative reagent and a total of 1,053 effective samples were tested. The kit tested positive, negative, and overall compliance rates were 100% and Kappa values were 1.000 (p=0.00), the consistency of the two reagents for the detection was good.

[Precautions]

1. Please read this manual carefully before testing. Operator should undergo professional technical training. Signal counting personnel must be able to observe orange and green signals.

2. When testing clinical samples, the test will not provide any test results when the hybridization signal is difficult to count and the sample is not sufficient for repeated retests. If the amount of cells is not sufficient for analysis, the test will not provide test results.

3. The xylene, formamide, and DAPI dye used in this experiment are potentially toxic or carcinogenic and should be handled in a fume hood. Wear masks and gloves to avoid direct contact.

4. The results of this kit will be affected by various factors of the sample itself, as well as restrictions such as enzyme digestion time, hybridization temperature and time, operating environment, and limitations of current molecular biology techniques, which may result in the wrong amplification of HER2 gene.

[Reference]

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[3] Zhao Yanmiao, Liu Xinlan, Qin Zheng. FISH and immunohistochemistry for the clinical value of Her-2 gene and protein in breast cancer[J]. NINGXI MEDICAL JOURNAL. 2013, 35(7): 586-89.

[4] Zheng YH, Yang YH, Qiu XW. Comparison of IHC and FISH detection methods for HER2 gene in breast cancer and its relationship with clinical pathology[J]. Journal of Fujian Medical University, 2013,47(5):293-98.

[Basic information]

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