



## ZytoDot 2C SPEC ERBB2/CEN 17 Probe Kit

REF C-3022-10  $\Sigma$  10

REF C-3022-40  $\Sigma$  40

For the qualitative detection of human ERBB2 gene amplifications and alpha satellites of chromosome 17 by chromogenic *in situ* hybridization (CISH)



In vitro diagnostic medical device  
according to EU directive 98/79/EC

### 1. Intended use

The ZytoDot 2C SPEC ERBB2/CEN 17 Probe Kit is intended to be used for the qualitative detection of amplifications involving the human ERBB2 gene as well as the detection of chromosome 17 alpha satellites in formalin-fixed, paraffin-embedded specimens by chromogenic *in situ* hybridization (CISH).

Interpretation of the results must be made within the context of the patient's clinical history with respect to further clinical and pathologic data of the patient by a qualified pathologist.

### 2. Clinical relevance

The ERBB2 gene (a.k.a. HER2 and NEU) is located in the chromosomal region 17q12 and encodes the cellular growth factor receptor p185. Amplification of the proto-oncogene ERBB2, observed in approximately 20% of all breast cancer samples, has been correlated with a poor prognosis of the disease. Similar results have been obtained for a variety of other malignant neoplasms e.g. ovarian cancer, stomach cancer, and carcinomas of the salivary gland.

### 3. Test principle

The chromogenic *in situ* hybridization (CISH) technique allows the detection and visualization of specific nucleic acid sequences in cell preparations. Hapten-labeled nucleotide fragments, so called CISH probes, and their complementary target sequences in the preparations are co-denatured and subsequently allowed to anneal during hybridization. Afterwards, unbound and unbound probe fragments are removed by stringency washing steps. Duplex formation of the labeled probe can be visualized using primary (unmarked) antibodies, which are detected by secondary polymerized enzyme-conjugated antibodies. The enzymatic reaction with chromogenic substrates leads to the formation of colored precipitates. After counterstaining the nucleus with a nuclear dye, hybridized probe fragments are visualized by light microscopy.

### 4. Reagents provided

The ZytoDot 2C SPEC ERBB2/CEN 17 Probe Kit is available in two sizes and is composed of:

Code	Component	Quantity		Container
		40 $\Sigma$	10	
PT2	Heat Pretreatment Solution EDTA	500 ml	150 ml	Screw-cap bottle (large)
ES1	Pepsin Solution	4 ml	1 ml	Dropper bottle, white cap
PD12	ZytoDot 2C SPEC ERBB2/CEN 17 Probe	0.4 ml	0.1 ml	Reaction vessel, brown lid
WB1	Wash Buffer SSC	560 ml	210 ml	Screw-cap bottle (large)
WB5	20x Wash Buffer TBS	2x 50 ml	50 ml	Screw-cap bottle
AB14	Anti-DIG/DNP-Mix	4 ml	1 ml	Dropper bottle, yellow cap
AB13	HRP/AP-Polymer-Mix	4 ml	1 ml	Dropper bottle, blue cap
SB6a	AP-Red Solution A	0.4 ml	0.1 ml	Dropper bottle, red cap (small)
SB6b	AP-Red Solution B	15 ml	4 ml	Dropper bottle, red cap
SB7a	HRP-Green Solution A	0.8 ml	0.2 ml	Dropper bottle, green cap (small)
SB7b	HRP-Green Solution B	15 ml	4 ml	Dropper bottle, green cap
CS2	Nuclear Blue Solution	20 ml	4 ml	Screw-cap bottle, black
MT4	Mounting Solution (alcoholic)	4 ml	1 ml	Glass bottle, brown
	AP-Red reaction vessel	2	1	Graduated cup, red lid
	HRP-Green reaction vessel	2	1	Graduated cup, green lid
	Instructions for use	1	1	

**C-3022-10 (10 tests):** Components **PD12**, **ES1**, **AB14**, **AB13**, **SB6a-b**, **SB7a-b**, **CS2**, and **MT4** are sufficient for 10 reactions. Component **PT2** is sufficient for 2 staining jars of 70 ml each. Component **WB1** is sufficient for 3 staining jars of 70 ml each. Component **WB5** is sufficient for 14 staining jars of 70 ml each.

**C-3022-40 (40 tests):** Components **PD12**, **ES1**, **AB14**, **AB13**, **SB6a-b**, **SB7a-b**, **CS2**, and **MT4** are sufficient for 40 reactions. Component **PT2** is sufficient for 7 staining jars of 70 ml each. Component **WB1** is sufficient for 8 staining jars of 70 ml each. Component **WB5** is sufficient for 28 staining jars of 70 ml each.

The ZytoDot 2C SPEC ERBB2/CEN 17 Probe (**PD12**) is composed of:

- Digoxigenin-labeled polynucleotides (~1.1 ng/ $\mu$ l), which target sequences mapping in 17q12\* (chr17:37,725,661-37,973,541) harboring the ERBB2 gene region (see Fig. 1).
- Dinitrophenyl-labeled polynucleotides (~1.1 ng/ $\mu$ l), which target sequences mapping in 17p11.1-q11.1 specific for the alpha satellite centromeric region D17Z1 of chromosome 17.
- Formamide based hybridization buffer

\*according to Human Genome Assembly GRCh37/hg19

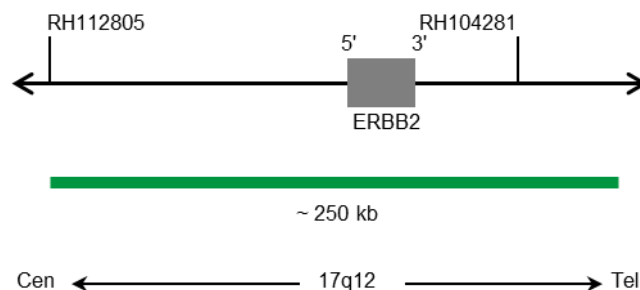


Fig. 1: SPEC ERBB2 Probe map (not to scale)

## 5. Materials required but not provided

- Positive and negative control specimens
- Microscope slides, positively charged
- Water bath (80°C, 98°C)
- Hybridizer or hot plate
- Hybridizer or humidity chamber in hybridization oven
- Adjustable pipettes (10 µl, 1000 µl)
- Staining jars or baths
- Timer
- Calibrated thermometer
- Ethanol or reagent alcohol
- Xylene
- Methanol 100%
- Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) 30%
- Deionized or distilled water
- Coverslips (22 mm x 22 mm, 24 mm x 32 mm)
- Rubber cement, e.g., Fixogum Rubber Cement (Prod. No. E-4005-50/-125) or similar
- Adequately maintained light microscope (400-630x)

## 6. Storage and handling

Store at 2-8°C in an upright position. Return to storage conditions immediately after use. Do not use reagents beyond expiry date indicated on the label. The product is stable until expiry date indicated on the label when handled accordingly.

## 7. Warnings and precautions

- Read the instructions for use prior to use!
- Do not use the reagents after the expiry date has been reached!
- This product contains substances (in low concentrations and volumes) that are harmful to health and potentially infectious. Avoid any direct contact with the reagents. Take appropriate protective measures (use disposable gloves, protective glasses, and lab garments)!
- Report any serious incident that has occurred in relation to the product to the manufacturer and the competent authority according to local regulations!
- If reagents come into contact with skin, rinse skin immediately with copious amounts of water!
- A material safety data sheet is available on our homepage ([www.zytovision.com](http://www.zytovision.com)).
- Do not reuse reagents, unless reuse is explicitly permitted!
- Avoid any cross-contamination and micro-bacterial contamination of the reagents!
- The specimens must not be allowed to dry during the hybridization and washing steps!

### Special labeling of ES1:

EUH208	Contains Pepsin A. May produce an allergic reaction.
EUH210	Safety data sheet available on request.

### Hazard and precautionary statements for AB13, AB14, PT2, SB7b, WB1 and WB5:

The hazard-determining component is a mixture of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC no. 247-500-7] and 2-methyl-2H-isothiazol-3-one [EC no. 220-239-6] (3:1).



#### Warning

H317	May cause an allergic skin reaction.
P261	Avoid breathing dust/fume/gas/mist/vapours/spray.
P272	Contaminated work clothing should not be allowed out of the workplace.
P280	Wear protective gloves/protective clothing/eye protection/face protection.
P302+P352	IF ON SKIN: Wash with plenty of water.
P333+P313	IF skin irritation or rash occurs: Get medical advice/attention.
P362+P364	Take off contaminated clothing and wash it before reuse.

### Hazard and precautionary statements for SB7a:

The hazard-determining components are methanol and hydrogen peroxide solution 30 %.



#### Danger

H225	Highly flammable liquid and vapour.
H301+H311 +H331	Toxic if swallowed, in contact with skin or if inhaled.
H370	Causes damage to organs.
P210	Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.
P233	Keep container tightly closed.
P260	Do not breathe dust/fume/gas/mist/vapours/spray.
P280	Wear protective gloves/protective clothing/eye protection/face protection.
P308+P311	IF exposed or concerned: Call a POISON CENTER/doctor.
P403+P235	Store in a well-ventilated place. Keep cool.

### Hazard and precautionary statements for PD12:

The hazard-determining component is formamide.



#### Danger

H351	Suspected of causing cancer.
H360FD	May damage fertility. May damage the unborn child.
H373	May cause damage to organs through prolonged or repeated exposure.
P201	Obtain special instructions before use.
P202	Do not handle until all safety precautions have been read and understood.
P260	Do not breathe dust/fume/gas/mist/vapours/spray.
P280	Wear protective gloves/protective clothing/eye protection/face protection.
P308+P313	IF exposed or concerned: Get medical advice/attention.
P405	Store locked up.

**Hazard and precautionary statements for CS2:**

The hazard-determining component is ethanediol, ethylene glycol.

**Warning**

- H373 May cause damage to organs through prolonged or repeated exposure.  
 P260 Do not breathe dust/fume/gas/mist/vapours/spray.  
 P314 Get medical advice/attention if you feel unwell.

**Hazard and precautionary statements for MT4:**

The hazard-determining component is xylene.

**Warning**

- H226 Flammable liquid and vapour.  
 H312+H332 Harmful in contact with skin or if inhaled.  
 H315 Causes skin irritation.  
 H319 Causes serious eye irritation.  
 H335 May cause respiratory irritation.  
 H373 May cause damage to organs through prolonged or repeated exposure.  
 P210 Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.  
 P260 Do not breathe dust/fume/gas/mist/vapours/spray.  
 P280 Wear protective gloves/protective clothing/eye protection/face protection.  
 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 advice/attention.  
 P403+P235 Store in a well-ventilated place. Keep cool.  
 EUH208 Contains methyl 2-methylprop-2-enoate; methyl 2-methylpropenoate; methyl methacrylate. May produce an allergic reaction.

**8. Limitations**

- For *in vitro* diagnostic use.
- For professional use only.
- For non-automated use only.
- The clinical interpretation of any positive staining, or its absence, must be done within the context of clinical history, morphology, other histopathological criteria as well as other diagnostic tests. It is the responsibility of a qualified pathologist to be familiar with the CISH probes, reagents, diagnostic panels, and methods used to produce the stained preparation. Staining must be performed in a certified, licensed laboratory under the supervision of a pathologist who is responsible for reviewing the stained slides and assuring the adequacy of positive and negative controls.
- Specimen staining, especially signal intensity and background staining, is dependent on the handling and processing of the specimen prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, sectioning, or contamination with other specimens or fluids may produce artefacts or false results. Inconsistent results may result from variations in fixation and embedding methods, as well as from inherent irregularities within the specimen.
- The probe should be used only for detecting loci described in chapter 4. "Reagents provided".
- The performance was validated using the procedures described in these instructions for use. Modifications to these procedures might alter the performance and have to be validated by the user.

**9. Interfering substances**

The following fixatives are incompatible with ISH:

- Bouin's fixative
- B5 fixative
- Acidic fixatives (e.g., picric acid)
- Zenker's fixative
- Alcohols (when used alone)
- Mercuric chloride
- Formaldehyde/zinc fixative
- Hollande's fixative
- Non-buffered formalin

**10. Preparation of specimens**

Recommendations:

- Avoid cross-contamination of samples in any step of preparation as this may lead to erroneous results.
- Fixation in 10% neutrally buffered formalin for 24 h at room temperature (18-25°C).
- Sample size  $\leq 0.5 \text{ cm}^3$ .
- Use premium quality paraffin.
- Embedding should be carried out at temperatures lower than 65°C.
- Prepare 3-5  $\mu\text{m}$  microtome sections.
- Use positively charged microscope slides.
- Fix tissue sections for 2-16 h at 50-60°C.

**11. Preparatory treatment of the device**

20x Wash Buffer TBS (WB5) is to be prepared according to the instructions in 12. "Assay procedure". All other kit reagents are ready-to-use. No reconstitution, mixing, or dilution is required. Bring probe to room temperature (RT) and mix briefly before use.

**12. Assay procedure****12.1 Day 1****Preparatory steps**

- Prepare an ethanol series (70%, 90%, and 100% ethanol solutions):* Dilute 100% ethanol with deionized or distilled water. These solutions can be stored in suitable containers and can be re-used.
- Heat Pretreatment Solution EDTA (PT2):* Heat to 98°C in a covered staining jar.
- Preparation of 3% H<sub>2</sub>O<sub>2</sub>:* Dilute 1 part 30% H<sub>2</sub>O<sub>2</sub> in 9 parts 100% methanol.
- ZytoDot 2C CISH Probe:* Bring to room temperature before use.

**Pre-treatment (dewax/proteolysis)**

- Incubate slides for 10 min at 70°C (e.g., on a hot plate).
- Incubate slides for 2x 5 min in xylene.
- Incubate slides for 3x 3 min in 100% ethanol.
- Incubate slides for 5 min in 3% H<sub>2</sub>O<sub>2</sub>.
- Wash slides 2x 1 min in deionized or distilled water.
- Incubate for 15 min in pre-warmed Heat Pretreatment Solution EDTA (PT2) at 98°C.

*Use eight slides per staining jar (add dummy slides if needed).*

- Transfer slides immediately to deionized or distilled water and wash for 2x 2 min.
- Apply (dropwise) Pepsin Solution (ES1) to the specimen and incubate for 5-15 min at 37°C in a humidity chamber.

*ES1 may form precipitates, which do not affect the quality.*

*As a general rule, we recommend to ascertain the optimum time for proteolysis in pre-tests.*

- Immerse slides in deionized or distilled water.
- Dehydration in: 70%, 90%, and 100% ethanol, each for 1 min.
- Air dry sections.

*Note: Make sure to completely dry sections prior to probe application.*

**Denaturation and hybridization**

1. Pipette 10  $\mu$ l of the probe onto each pretreated specimen.
2. Cover specimens with a 22 mm x 22 mm coverslip (avoid trapped bubbles) and seal the coverslip.

We recommend using rubber cement (e.g., Fixogum) for sealing.

3. Place slides on a hot plate or hybridizer and denature specimens for 5 min at 79°C.
4. Transfer slides to a humidity chamber and hybridize overnight at 37°C (e.g., in a hybridization oven).

It is essential that specimens do not dry out during the hybridization step.

**12.2 Day 2****Preparatory steps**

1. Wash Buffer SSC (WB1): For stringency wash, heat to 80°C in a covered staining jar. **WB1** may form precipitates at 2-8°C, which do not affect the quality and should dissolve when heated.
2. Preparation of 1x Wash Buffer TBS: Dilute 1 part of 20x Wash Buffer TBS (WB5) in 19 parts deionized or distilled water.

Diluted 1x Wash Buffer TBS is stable for one week when stored at 2-8°C.

3. Anti-DIG/DNP-Mix (AB14), HRP/AP-Polymer-Mix (AB13), AP-Red Solution A (SB6a), AP-Red Solution B (SB6b), HRP-Green Solution A (SB7a), HRP-Green Solution B (SB7b), Nuclear Blue Solution (CS2), Mounting Solution (alcoholic) (MT4): Bring to room temperature before use.

Components **SB7a** and **SB7b** may form precipitates, which do not affect the staining quality.

**Post-hybridization and detection**

1. Carefully remove the rubber cement or glue.
2. Remove the coverslip by submerging the slides in Wash Buffer SSC (WB1) at room temperature for 5 min.

**WB1** can be reused once. Store at 2-8°C for a maximum of one week.

3. Wash slides for 5 min in Wash Buffer SSC (WB1) at 80°C.

Use eight slides per staining jar (add dummy slides if needed).

4. Wash slides 2x 1 min in deionized or distilled water.
5. Immerse slides in 1x Wash Buffer TBS.
6. Apply Anti-DIG/DNP-Mix (AB14) (1-2 drops per slide) to the slides and incubate for 15 min at 37°C in a humidity chamber.
7. Wash slides 3x 1 min in 1x Wash Buffer TBS.
8. Apply HRP/AP-Polymer-Mix (AB13) (1-2 drops per slide) to the slides and incubate for 15 min at 37°C in a humidity chamber.
9. Wash slides 3x 1 min in 1x Wash Buffer TBS.
10. Prepare AP-Red Solution (working solution): fill 1 ml AP-Red Solution B (**SB6b**) in a graduated cup and add one drop (30  $\mu$ l) of AP-Red Solution A (**SB6a**). Mix well.
11. Apply AP-Red Solution (1-2 drops per slide) to the slides and incubate for 10 min at RT.
12. During the incubation, prepare HRP-Green Solution (working solution): fill 1 ml HRP-Green Solution B (SB7b) in a graduated cup and add two drops (2x 20  $\mu$ l) HRP-Green Solution A (SB7a). Mix well.
13. Wash slides for 2 min in deionized or distilled water.
14. Apply HRP-Green Solution dropwise (1-2 drops per slide) to the slides and incubate for 10 min at room temperature.
15. Wash slides for 2 min in deionized or distilled water.
16. Counterstain specimens for 2 min with Nuclear Blue Solution (CS2).
17. Transfer slides into a staining jar and wash 2 min under cold running tap water.
18. Dehydrate 3x 30 s in 100% ethanol (use very pure ethanol).
19. Incubate slides for 2x 30 s in xylene (use very pure xylene).

Do not prolong or shorten the incubation time as this might result in loss of signals!

20. Avoiding trapped bubbles, cover the samples with a coverslip (22 mm x 22 mm; 24 mm x 32 mm) by using Mounting Solution (alcoholic) (MT4). Allow 20-30 min for the coverslip to become immobilized.

Using a pipette tip which has been cut off to increase the size of the opening, can make the pipetting process easier.

21. Evaluate stained specimens by using light microscopy.

**13. Interpretation of results**

Hybridization signals of Digoxigenin-labeled polynucleotides appear as dark green colored distinct dots (ERBB2 gene region), and Dinitrophenyl-labeled polynucleotides appear as bright red colored distinct dots (CEN 17).

**Normal situation:** In interphases of normal cells or cells without an amplification involving the ERBB2 gene region, two distinct dot-shaped green signals and two distinct dot-shaped red signals appear (see Fig. 2).

**Aberrant situation:** In cells with an amplification of the ERBB2 gene region, an increased number of green signal or green signal clusters will be observed (see Fig. 2).

Overlapping signals may appear as brown signals.

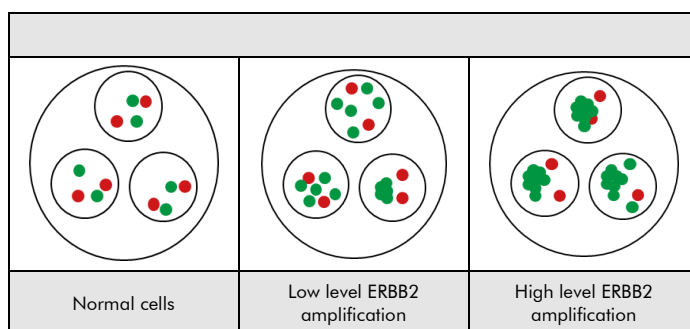


Fig. 2: Expected results in normal and aberrant nuclei

Other signal patterns than those described above may be observed in some abnormal samples. These unexpected signal patterns should be further investigated.

**Please note:**

- Due to decondensed chromatin, single CISH signals can appear as small signal clusters. Thus, two or three signals of the same size, separated by a distance  $\leq$  1 signal diameter, should be counted as one signal.
- Prior to signal enumeration, the specimen should be scanned for any possible intratumoral heterogeneity at 100- to 200-fold magnification.
- Visualization of signals should be performed at least at 400-fold magnification resulting in easily visible signals. A 630-fold magnification is recommended for probes detecting chromosomal breaks. Do not use contrast enhancing filter lenses as this might distort the signal color. To obtain signals in bright colors, open the aperture diaphragm. Be sure to focus up and down when evaluating a nucleus, as red and green signals might be located on top of each other.
- Do not evaluate areas of necrosis, overlapping nuclei, over-digested nuclei and nuclei with weak signal intensity.
- Due to mitosis, additional signals may be visible even in a small percentage of non-neoplastic cells. Occasionally, nuclei with missing signals may be observed in paraffin-embedded specimens due to cutting artefacts.
- A negative or unspecific result can be caused by multiple factors (see chapter 17. "Troubleshooting").
- In order to correctly interpret the results, the user must validate this product prior to use in diagnostic procedures according to national and/or international guidelines.

### 14. Recommended quality control procedures

In order to monitor correct performance of processed specimens and test reagents, each assay should be accompanied by internal and external controls. If internal and/or external controls fail to demonstrate appropriate staining, results with patient specimens must be considered invalid.

**Internal control:** Non-neoplastic cells within the specimen that exhibit normal signal pattern, e.g., fibroblasts.

**External control:** Validated positive and negative control specimens.

### 15. Performance characteristics

The performance of the probe was determined by comparison against the corresponding IVD approved FISH probe. The concordance was 100%.

**Accuracy:** The accuracy was calculated as 100%.

**Analytical sensitivity:** The analytical sensitivity was calculated as 100%.

**Analytical specificity:** The analytical specificity was calculated as 100%.

### 16. Disposal

The disposal of reagents must be carried out in accordance with local regulations.

### 17. Troubleshooting

Any deviation from the operating instructions can lead to inferior staining results or to no staining at all.

#### Weak signals or no signals at all

Possible cause	Action
Cell or tissue sample has not been properly fixed	Optimize fixing time and fixative
Heat pretreatment, proteolysis, hybridization, denaturation, stringency wash or antibody-incubation temperature not correct	Check temperature of all technical devices used, using a calibrated thermometer. Use always the same number of slides in solutions with critical temperature
Proteolytic pretreatment not carried out properly	Depending on multiple factors, e.g., nature and duration of fixing, thickness of sections, and nature of tissue/cells, different incubation times may be required. Ascertain the optimum time for pepsin incubation in pre-tests
Hybridization time too short	Hybridize for at least 12 h; extend hybridization time if necessary
Old dehydration solutions	Prepare fresh dehydration solutions
Probe evaporation	When using a hybridizer, the use of the wet stripes/water filled tanks is mandatory. When using a hybridization oven, the use of a humidity chamber is required. In addition, the coverslip should be sealed completely, e.g., with Fixogum, to prevent drying-out of the sample during hybridization
Incubation with chromogenic substrate too short	Extend incubation time
Counterstaining time too long	The counterstaining time depends on the nature of the specimen and should be optimized accordingly. Avoid dark counterstaining, because it may obscure positive staining signals
Bluing of counterstain not carried out properly	Use cold running tap water for bluing; do not use warm or hot water, or bluing reagents

#### Signals too strong

Possible cause	Action
Proteolytic pretreatment carried out too long	Depending on multiple factors, e.g., nature and duration of fixing, thickness of sections, and nature of tissue/cells, different incubation times may be required. Ascertain the optimum time for pepsin incubation in pre-tests
AP-Red Solution incubation time not correct	If required, the incubation time can be shortened down to 5 min. Do not heat substrate solution over 25°C; incubate at room temperature only
HRP-Green solution incubation time not correct	If required, the incubation time can be shortened down to 7 min. Do not heat substrate solution over 25°C; incubate at room temperature only

#### Red signals too weak

Possible cause	Action
AP-Red Solution was exposed to strong direct light	Prepare and use AP-Red Solution protected from strong direct light
AP-Red Solution was prepared too early	Prepare prior to immediate use
AP-Red Solution incubation time not correct	If required, the incubation time can be extended up to 15 min
Insufficient preparation of chromogenic substrate	Do not increase volume of Solution A

#### Green signals too weak

Possible cause	Action
Incubation time of any washing steps after staining with HRP-Green too long	Do not exceed given incubation times
HRP-Green solution incubation time not correct	If required, the incubation time can be extended up to 15 min
Insufficient preparation of chromogenic substrate	Do not increase volume of Solution A

#### Signals fade or merge

Possible cause	Action
An unsuitable mounting solution has been used	Use only the mounting solution provided with the kit or xylene-based mounting solutions free of any impurities; do not use coverslip tape
Sections were not dehydrated properly	Use fresh ethanol and xylene solutions; use only xylene of "pure" quality

#### Uneven or in some parts only very light staining

Possible cause	Action
Incomplete dewaxing	Use fresh solutions; check duration of dewaxing times
Reagent volume too small	Ensure that the reagent volume is large enough to cover the tissue area
Air bubbles caught before hybridization or during mounting	Avoid air bubbles

#### Inconsistent results

Possible cause	Action
Insufficient drying before probe application	Extend air-drying

Too much water/wash buffer on tissue prior to application of pepsin, antibodies and/or color substrates	Ensure that excess liquid is removed from tissue section by blotting or shaking it off the slide. Small amounts of residual water/wash buffer do not interfere with the test
Variations in tissue fixation and embedding methods	Optimize fixation and embedding methods
Variations in tissue section thickness	Optimize sectioning

**Morphology degraded**

Possible cause	Action
Cell or tissue sample has not been properly fixed	Optimize fixing time and fixative
Proteolytic pretreatment not carried out properly	Optimize pepsin incubation time; decrease if necessary

**Cross hybridization signals; noisy background**

Possible cause	Action
Stringency wash temperature not correct	Check temperature of the technical devices used, using a calibrated thermometer. Use always the same number of slides in the jar. We recommend not to use more than eight slides per jar for heat incubation steps
Slides not thoroughly rinsed	Use fresh and sufficient wash buffer and deionized or distilled water where indicated
Sections dried out any time during or after hybridization	Avoid sections being dried out; use humidity chamber; seal coverslip properly
Prolonged substrate incubation time	Shorten substrate incubation time
Incomplete dewaxing	Use fresh solutions; check duration of dewaxing
Proteolytic pretreatment too strong	Optimize pepsin incubation time
Slides cooled to room temperature before hybridization	Transfer the slides quickly to hybridization temperature

**Overlapping signals**

Possible cause	Action
Inappropriate thickness of tissue sections	Prepare 3-5 $\mu\text{m}$ microtome sections

**Specimen floats off the slide**

Possible cause	Action
Unsuitable slide coating	Use appropriate (positively charged) slides
Proteolytic pretreatment too strong	Shorten pepsin incubation time

**18. Literature**

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**19. Revision**

Please refer to [www.zytovision.com](http://www.zytovision.com) for the most recent instructions for use as well as for instructions for use in different languages.

Our experts are available to answer your questions.  
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