



## ZytoDot SPEC ERBB2 Probe Kit

REF C-3003-40  $\Sigma$  40

For the qualitative detection of human ERBB2 gene amplifications by chromogenic *in situ* hybridization (CISH)



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

### 1. Intended use

The *ZytoDot* SPEC ERBB2 Probe Kit is intended to be used for the qualitative detection of human ERBB2 gene amplifications in formalin-fixed, paraffin-embedded specimens such as human breast cancer or gastric cancer tissues 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, unspecific 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* SPEC ERBB2 Probe Kit is available in one size and is composed of:

Code	Component	Quantity	Container
		$\Sigma$ 40	
PT2	Heat Pretreatment Solution EDTA	500 ml	Screw-cap bottle (large)
ES1	Pepsin Solution	4 ml	Dropper bottle, white cap
PD1	<i>ZytoDot</i> SPEC ERBB2 Probe	0.4 ml	Reaction vessel, brown lid
WB1	Wash Buffer SSC	560 ml	Screw-cap bottle (large)
WB4	PBS/Tween	2	Aluminum pack
BS1	Blocking Solution	4 ml	Dropper bottle, orange cap
AB1	Mouse-Anti-DIG	4 ml	Dropper bottle, pink cap
AB2	Anti-Mouse-HRP-Polymer	4 ml	Dropper bottle, violet cap
SB1a	DAB Solution A	0.3 ml	Dropper bottle, green cap
SB1b	DAB Solution B	10 ml	Dropper bottle, grey cap
CS1	Mayer's Hematoxylin Solution	20 ml	Screw-cap bottle, black
MT4	Mounting Solution (alcoholic)	4 ml	Glass bottle, brown
	Instructions for use	1	

**C-3003-40 (40 tests):** Components **PD1**, **ES1**, **BS1**, **AB1**, **AB2**, **SB1a-b**, **CS1**, 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 **WB4** is sufficient for 28 staining jars of 70 ml each.

The *ZytoDot* SPEC ERBB2 Probe (**PD1**) is composed of:

- Digoxigenin-labeled polynucleotides (~1.8 ng/ $\mu$ l), which target sequences mapping in 17q12\* (chr17:37,725,661-37,973,541) harboring the ERBB2 gene region (see Fig. 1).

- Formamide based hybridization buffer

\*according to Human Genome Assembly GRCh37/hg19

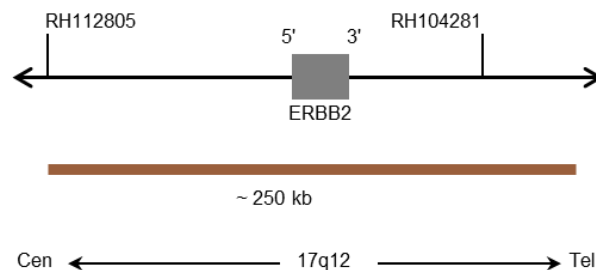


Fig. 1: SPEC ERBB2 Probe map (not to scale)

### 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  $\mu$ l, 1000  $\mu$ l)
- 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)!
- 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.

### Hazards and precautionary statements for BS1, AB1, AB2, PT2, and WB1:

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.

### Hazards and precautionary statements for SB1a:

The hazard-determining component is biphenyl-3,3',4,4'-tetrayltetraamine; diaminobenzidine.



#### Danger

H350	May cause cancer.
P201	Obtain special instructions before use.
P202	Do not handle until all safety precautions have been read and understood.
P280	Wear protective gloves/protective clothing/eye protection/face protection.
P308+P313	IF exposed or concerned: Get medical advice/attention.
P405	Store locked up.

### Hazards and precaution statements for SB1b:

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).



#### Danger

H317	May cause an allergic skin reaction.
H360D	May damage the unborn child.
P201	Obtain special instructions before use.
P202	Do not handle until all safety precautions have been read and understood.
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.
P362+P364	Take off contaminated clothing and wash it before reuse.
P308+P313	IF exposed or concerned: Get medical advice/attention.
P405	Store locked up.

### Hazards and precaution 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.

**Hazard and precautionary statements for PD1:**

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.

**8. Limitations**

- For *in vitro* diagnostic use.
- For professional 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 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**

PBS/Tween (WB4) is to be pretreated 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 (18-25°C) and mix briefly before use.

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

- (1) *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.
- (2) *Heat Pretreatment Solution EDTA (PT2):* Heat to 98°C in a covered staining jar.
- (3) *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.
- (4) *ZytoDot CISH Probe:* Bring to room temperature (RT) before use.

**Pretreatment (dewax/proteolysis)**

- (1) Incubate slides for 10 min at 70°C (e.g., on a hot plate).
- (2) Incubate slides for 2x 5 min in xylene.
- (3) Incubate slides for 3x 3 min in 100% ethanol.
- (4) Incubate slides for 5 min in 3% H<sub>2</sub>O<sub>2</sub>.
- (5) Wash slides 2x 1 min in deionized or distilled water.
- (6) 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).*

- (7) Transfer slides immediately to deionized or distilled water and wash for 2x 2 min.
- (8) 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.*

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

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

**Denaturation and hybridization**

- (1) Pipette 10  $\mu\text{l}$  of the ZytoDot CISH 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 94-95°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 Wash Buffer PBS/Tween: Add 1 tablet of PBS/Tween (WB4) to 1000 ml deionized or distilled water and dissolve.
- (3) Blocking Solution (BS1), Mouse-Anti-DIG (AB1), Anti-Mouse-HRP-Polymer (AB2), DAB Solution A (SB1a), DAB Solution B (SB1b), Mayer's Hematoxylin Solution (CS1), Mounting Solution (alcoholic) (MT4): Bring to room temperature before use.

### 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 Wash Buffer PBS/Tween.
- (6) Apply Blocking Solution (BS1) (1-2 drops per slide) to the slides and incubate for 10 min at RT.
- (7) Blot off Blocking Solution (BS1), **but do not rinse**
- (8) Apply Mouse-Anti-DIG (AB1) (1-2 drops per slide) to the slides and incubate for 30 min at RT.
- (9) Wash slides 3x 1 min in PBS/Tween.
- (10) Apply Anti-Mouse-HRP-Polymer (AB2) (1-2 drops per slide) to the slides and incubate for 30 min at RT.
- (11) Wash slides 3x 1 min in PBS/Tween.
- (12) Prepare DAB Solution (working solution): fill 1 ml DAB Solution B (SB1b) in a graduated cup and add one drop (30 µl) DAB Solution A (SB1a). Mix well.
- (13) Apply DAB Solution (1-2 drops per slide) to the slides and incubate for 30 min at RT.
- (14) Transfer slides into a staining jar and wash 2 min under cold running tap water.
- (15) Counterstain specimens for 5-10 sec with Mayer's Hematoxylin Solution (CS1).
- (16) Transfer slides into a staining jar and wash 2 min under cold running tap water.
- (17) Dehydration in: 70%, 90%, and 100% ethanol, each for 1 min.
- (18) Incubate slides for 2x 2 min in xylene (use very pure xylene).
- (19) 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.*

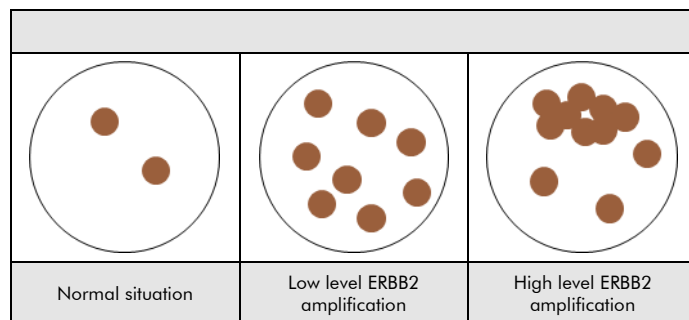
- (20) Evaluate stained specimens by using light microscopy.

### 13. Interpretation of results

The hybridization signals of Digoxigenin-labeled polynucleotides appear brown- to dark brown (ERBB2 gene region).

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

**Aberrant situation:** In cells with an amplification of the ERBB2 gene region or polysomy of chromosome 17, an increased number of the brown signal or brown signal clusters will be observed (see Fig. 2).



**Fig. 2: Expected results in normal and aberrant nuclei**

Other signal distribution may be observed in some abnormal samples which might result in a different signal pattern than described above, indicating variant rearrangements. 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- to 630-fold magnification resulting in easily visible signals.
- 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 artifacts.
- 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
Insufficient preparation of chromogenic substrate	Instead of using one drop of DAB Solution A use 30 $\mu$ l
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
Substrate reaction is too intense	Shorten substrate incubation time; do not heat substrate solution over 25°C; incubate at room temperature only

**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

**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

**Tissue 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; increase or 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 $\mu$ 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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Our experts are available to answer your questions.  
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