CLC patients carrying EML4
sent a very
characteristic
result in a fusion with the
EML4 gene. This gene exerts charac-
teristic activities through fusion
with the ALK gene, which target
sequences mapping in 2p23.2 (chr2:29,460,144-29,681,581)
proximal to the ALK breakpoint region (see Fig. 1).

Additionally, translocations affecting the ALK
locus are frequently detected in non-
small cell lung cancer (NSCLC) and lead to the formation of EML4-ALK
fusion transcripts. ALK kinase targeted therapies may represent a very
effective therapeutic strategy in NSCLC patients carrying EML4-ALK
fusion transcripts.

1. Intended use
The *ZytoDot 2C SPEC ALK Break Apart Probe* (PD35) is intended to be
used for the qualitative detection of translocations involving the human ALK
gene at 2p23.2 in formalin-fixed, paraffin-embedded specimens by
chromogenic *in situ* hybridization (CISH). The probe is intended to be used
in combination with the *ZytoDot 2C CISH Implementation Kit*
(Prod. No. C-3044-10/-40).

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 ALK (ALK receptor tyrosine kinase, a.k.a. CD246) gene encodes a
transmembrane receptor tyrosine kinase. This gene exerts characteristic
oncogenic activities through fusion to several gene partners or mutations
both in hematopoietic and non-hematopoietic solid tumors. Translocations
affecting the ALK gene locus are frequently found in anaplastic large cell
lymphoma (ALCL), an aggressive non-Hodgkin lymphoma arising from T-
cells. The most frequent translocation t(2;5) results in a fusion with the
NPM1/ALK fusion protein, which is constitutively activated through
autophosphorylation, and that in turn mediates malignant cell
transformation by activating downstream effectors like e.g. STAT3.
Additionally, inversions affecting the ALK gene located on the short arm of
chromosome 2 (inv(2)(p21p23)) have been frequently detected in non-
small cell lung cancer (NSCLC) and lead to the formation of EML4-ALK
fusion transcripts. ALK kinase targeted therapies may represent a very
effective therapeutic strategy in NSCLC patients carrying EML4-ALK
rearrangements.

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 2C SPEC ALK Break Apart Probe* is composed of:

- Digoxigenin-labeled polynucleotides (~0.50 ng/µl), which target
  sequences mapping in 2p23.2* (chr2:29,460,144-29,681,581)
  proximal to the ALK breakpoint region (see Fig. 1).
- Dinitrophenyl-labeled polynucleotides (~0.75 ng/µl), which target
  sequences mapping in 2p23.2* (chr2:29,174,204-29,383,335)
  distal to the ALK breakpoint region (see Fig. 1).
- Formamide based hybridization buffer
  *according to Human Genome Assembly GRCh37/hg19

5. Materials required but not provided
- *ZytoDot 2C CISH Implementation Kit* (Prod. No. C-3044-10/-40)
  - 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 (H2O2) 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).
- 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!

Hazard and precautionary statements:

The hazard-determining component is formamide.

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
- Holland’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 ≤ 0.5 cm².
- Use premium quality paraffin.
- Embedding should be carried out at temperatures lower than 65°C.
- Prepare 3-5 µm microtome sections.
- Use positively charged microscope slides.
- Fix tissue sections for 2-16 h at 50-60°C.

11. Preparatory treatment of the device

The product is 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

**Specimen pretreatment**

Perform specimen pretreatment (e.g., dewaxing, proteolysis) according to the instructions for use of the ZytoDot 2C CISH Implementation Kit.

**Denaturation and hybridization**

1. Pipette 10 µl 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.

**Post-hybridization**

Perform post-hybridization processing (washing, detection, counter-staining, mounting, microscopy) according to the instructions for use of the ZytoDot 2C CISH Implementation Kit.

13. Interpretation of results

Using the ZytoDot 2C CISH Implementation Kit, hybridization signals of Digoxygenin-labeled polynucleotides appear as dark green colored distinct dots (proximal to the ALK breakpoint region), and Dinitrophenyl-labeled polynucleotides appear as bright red colored distinct dots (distal to the ALK breakpoint region).

**Normal situation:** In interphases of normal cells or cells without a translocation involving the ALK gene region, two red/green fusion signals appear (see Fig. 2).

**Aneurorant situation:** One ALK gene region affected by a translocation is indicated by one separate green signal and one separate red signal. EML4-ALK inversion with deletion of 5'-ALK sequences is indicated by one or multiple isolated red signals (see Fig. 2).

Overlapping signals may appear as brown signals.
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

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell or tissue sample has not been properly fixed</td>
<td>Optimize fixation time and fixative</td>
</tr>
<tr>
<td>Heat pretreatment, proteolysis, hybridization, denaturation, stringency wash or antibody-incubation temperature not correct</td>
<td>Check temperature of all technical devices used, using a calibrated thermometer. Use always the same number of slides in solutions with critical temperature</td>
</tr>
<tr>
<td>Proteolytic pretreatment not carried out properly</td>
<td>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</td>
</tr>
<tr>
<td>Hybridization time too short</td>
<td>Hybridize for at least 12 h; extend hybridization time if necessary</td>
</tr>
<tr>
<td>Old dehydration solutions</td>
<td>Prepare fresh dehydration solutions</td>
</tr>
<tr>
<td>Probe evaporation</td>
<td>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 Fixagum, to prevent drying-out of the sample during hybridization</td>
</tr>
<tr>
<td>Incubation with chromogenic substrate too short</td>
<td>Extend incubation time</td>
</tr>
<tr>
<td>Counterstaining time too long</td>
<td>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</td>
</tr>
<tr>
<td>Bluing of counterstain not carried out properly</td>
<td>Use cold running tap water for bluing; do not use warm or hot water, or bluing reagents</td>
</tr>
</tbody>
</table>

### Signals too strong

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteolytic pretreatment carried out too long</td>
<td>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</td>
</tr>
<tr>
<td>AP-Red Solution incubation time not correct</td>
<td>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</td>
</tr>
<tr>
<td>HRP-Green solution incubation time not correct</td>
<td>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</td>
</tr>
</tbody>
</table>

### Red signals too weak

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-Red Solution was exposed to strong direct light</td>
<td>Prepare and use AP-Red Solution protected from strong direct light</td>
</tr>
<tr>
<td>AP-Red Solution was prepared too early</td>
<td>Prepare prior to immediate use</td>
</tr>
<tr>
<td>AP-Red Solution incubation time not correct</td>
<td>If required, the incubation time can be extended up to 15 min</td>
</tr>
</tbody>
</table>

### Genomic aberrations due to small deletions, duplications or inversions might result in inconspicuous signal patterns.

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 ≤ 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.
Inconsistent results

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insufficient drying before probe application</td>
<td>Extend air-drying</td>
</tr>
<tr>
<td>Too much water/wash buffer on tissue prior to application of pepsin, antibodies and/or color substrates</td>
<td>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</td>
</tr>
<tr>
<td>Variations in tissue fixation and embedding methods</td>
<td>Optimize fixation and embedding methods</td>
</tr>
<tr>
<td>Variations in tissue section thickness</td>
<td>Optimize sectioning</td>
</tr>
</tbody>
</table>

Morphology degraded

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell or tissue sample has not been properly fixed</td>
<td>Optimize fixing time and fixative</td>
</tr>
<tr>
<td>Proteolytic pretreatment not carried out properly</td>
<td>Optimize pepsin incubation time; decrease if necessary</td>
</tr>
</tbody>
</table>

Cross hybridization signals, noisy background

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stringency wash temperature not correct</td>
<td>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</td>
</tr>
<tr>
<td>Slides not thoroughly rinsed</td>
<td>Use fresh and sufficient wash buffer and deionized or distilled water where indicated</td>
</tr>
</tbody>
</table>

Signs fade or merge

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>An unsuitable mounting solution has been used</td>
<td>Use only the mounting solution provided with the kit or xylene-based mounting solutions free of any impurities; do not use coverslip tape</td>
</tr>
<tr>
<td>Sections were not dehydrated properly</td>
<td>Use fresh ethanol and xylene solutions; use only xylene of “pure” quality</td>
</tr>
</tbody>
</table>

Uneven or in some parts only very light staining

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

Green signals too weak

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

Sections dried out any time during or after hybridization

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolonged substrate incubation time</td>
<td>Shorten substrate incubation time</td>
</tr>
<tr>
<td>Incomplete dewaxing</td>
<td>Use fresh solutions; check duration of dewaxing</td>
</tr>
<tr>
<td>Proteolytic pretreatment too strong</td>
<td>Optimize pepsin incubation time</td>
</tr>
<tr>
<td>Slides cooled to room temperature before hybridization</td>
<td>Transfer the slides quickly to hybridization temperature</td>
</tr>
</tbody>
</table>

Overlapping signals

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inappropriate thickness of tissue sections</td>
<td>Prepare 3-5 µm microtome sections</td>
</tr>
</tbody>
</table>

Specimen floats off the slide

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsuitable slide coating</td>
<td>Use appropriate (positively charged) slides</td>
</tr>
<tr>
<td>Proteolytic pretreatment too strong</td>
<td>Shorten pepsin incubation time</td>
</tr>
</tbody>
</table>

18. Literature


19. Revision

Please refer to 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. Please contact helpltech@zytovision.com

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