

BIOGNOSTIK[®]



HybriProbe[™]

HybriProbe oligonucleotides and HybriBuffers are for research use only. Not for diagnostic use. Not for use in humans.

Spin tubes before opening. Vortex tubes always before use because diluted oligos concentrate at the bottom of the tube during thawing.

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Introduction

In Situ Hybridization

Introduced in the late sixties, *in situ* hybridization (ISH) has grown to become an increasingly important technique performed routinely in life science applications. With this technology, gene expression can be specifically visualized in preserved tissue sections or cells and thereby related to microanatomy – a significant advantage over Northern Blotting.

BIOGNOSTIK is the leading supplier of custom-made HybriProbe Kits for ISH worldwide. Innumerable renowned international scientists have profited greatly from *BIOGNOSTIK*'s unique sequence design expertise. Many publications presenting excellent results obtained with HybriProbes illustrate the success of our sequence design.

This HybriProbe manual describes and discusses all the important aspects of *in situ* hybridization and guides you through tissue preparation, fixation, mounting, prehybridization, hybridization, post-hybridization washes and signal detection.

BIOGNOSTIK's Kits for Non-Radioactive ISH

When you purchase a genuine HybriProbe Custom Design Kit you gain access to *BIOGNOSTIK*'s expertise in the field of *in situ* hybridization and benefit from their proven leadership position. *BIOGNOSTIK* has developed a unique technology for the design and manufacture of premium quality *in situ* hybridization kits. With these kits, you can determine the mRNA expression of any gene of interest conveniently, reliably and safely.

BIOGNOSTIK's HybriProbes are available in convenient ready-to-use kits. Each kit contains HybriProbes specific for the respective target mRNA along with different positive and negative control probes and HybriBuffer ISH. HybriProbes are highly specific for their targets.

By virtue of their sequence design, optimized length (on average 30 nucleotides) and high purity standards, HybriProbes exhibit excellent tissue penetration, high specificity and low background. These features are major factors in successful ISH.

All HybriProbes are designed to work under similar hybridization and washing conditions in conjunction with HybriBuffer ISH. This greatly facilitates the parallel detection of expression of different genes. HybriProbes have been used successfully on a wide variety of tissues.

Ready-Labelled HybriProbes

HybriProbes are ready FITC-labelled single-stranded phosphodiester DNA oligonucleotides. The double-FITC-labelled HybriProbes contain a fluorescein-isothiocyanate (FITC) group at the 5' and 3' nucleotides. FITC, an excellent immunogen equivalent to biotin and digoxigenin, has become the label of choice for non-radioactive detection of nucleic acids. Unlike biotin, FITC does not occur naturally in cells or tissues therefore endogenous signals of the tissue can be excluded.

Immunogenic detection has become a standardized procedure. Non-radioactive FITC-labelling ensures high stability and safe handling. HybriProbes are purified by HPLC via the labelling group. Therefore labelling ratio is virtually 100%. No further purification is necessary. HybriProbes can be added directly into the hybridization buffer. Due to their excellent properties, FITC-based detection systems are commercially available from different manufacturers.

HybriProbe Custom-Design TriSeq Kits

TriSeq Kits with three double FITC-labelled sequences directed at different regions of the same target mRNA are the new gold standard in ISH. Simultaneous use of three different HybriProbes against the same target mRNA enhances signal intensity and assay sensitivity. These kits are particularly suited to targeting mRNAs with low and normal expression.

The TriSeq kit components are:

- Three target-specific HybriProbes:
Specific HybriProbes directed against the optimal site of the mRNA of interest.
- Three random control HybriProbes:
Random control HybriProbes with comparable length and GC content. This probe serves as negative control for validating the specificity of a signal.
- Poly d(T) control:
Poly d(T) HybriProbe detects all mRNAs with a poly (A) tail. A homogeneous signal indicates appropriate and rapid fixation of the tissue.
- Beta-actin control:
Beta-actin is abundant in all cells. Thus it is ideal for testing the method and the mRNA's integrity.
- Alpha-tubulin control:
Alpha-tubulin is abundant in all cells. Thus it is ideal for testing the method and mRNA's integrity.
- HybriBuffer-ISH:
Ready-to-use buffer optimized for non-radioactive ISH.

HybriProbe Custom Design Kits

HybriProbe Custom Design Kits are most suitable for mRNA detection of genes with strong expression. These kits include one double-FITC-labelled HybriProbe and one random control. The positive and negative controls are the same as for the TriSeq kits.

The HybriProbe Custom Design Kit includes:
(A detailed description of the components is given above.)

- One specific HybriProbe
- One random control HybriProbe
- Poly d(T) control
- Beta-actin control
- Alpha-tubulin control
- HybriBuffer-ISH

HybriBuffer ISH

HybriBuffer ISH is an optimized ready-to-use buffer for *in situ* hybridization.

Its complex composition of DNA and RNA fractions, antioxidants, selected proteins, stabilizers and complexing macromolecules enhances hybridization efficacy and reduces nonspecific background.

This buffer contains formamide that reduces the incubation temperature to less than 40°C. With HybriProbes both the prehybridization and hybridization are routinely performed at 30°C. Similar hybridization conditions for all HybriProbes enable detection of different target mRNAs with the same protocol.

Immediately upon receipt store HybriBuffer ISH at -20°C. The buffer may be thawed and refrozen repeatedly. The thawed buffer may contain precipitates. Therefore, it should be heated up to 60°C until it is clear and without precipitates. Pipette the aliquots needed for prehybridization and hybridization into separate microtubes and refreeze the buffer stock as quickly as possible. The aliquots used for the ISH experiments should be heated to 95°C and cooled down on ice to 30-40°C to denature the double stranded buffer components directly before adding the HybriProbes.

Caution:

HybriBuffer ISH contains formamide. Formamide is toxic and may cause harm to the unborn child. Avoid exposure. Obtain special instructions before use. Avoid contact with skin or eyes. Wear suitable gloves. In case of an accident or if you feel unwell, seek medical advice immediately.

Storage and Stability

HybriProbes are diluted in sterile water at a concentration of 1 pmol/μl. One unit is equivalent to 3 μl or 3 pmol of the HybriProbes.

HybriProbes are stable at -20°C for at least 12 months. They may be thawed and refrozen several times. Always vortex before use because the probe settles at the bottom of the microtube during thawing.

HybriBuffer-ISH should be stored at -20°C. HybriBuffer-ISH can be thawed and refrozen several times. The buffer is stable at -20°C for at least 12 month.

Straightforward ISH Protocols

The following pages of this manual describe and discuss all important aspects of ISH including tissue preparation and fixation, mounting, prehybridization, hybridization, post-hybridization washes and detection.

HybriProbes are ready-to-use, so the procedures are very straightforward. Due to the high specificity of HybriProbes, hybridization can be performed at 30°C, which enables easy handling and optimal tissue preservation. HybriProbe Custom Design Kits are compatible with NBT/BCIP- or DAB-based FITC detection systems supplied by well-known manufacturers such as NEN, DAKO or Sigma (see Appendix C).

Getting Started

Control experiments with both positive and negative controls are very important for validating the specificity of the detection signal. We recommend that you start with the tissue control poly d(T) and technical controls beta-actin and alpha-tubulin. Both should give a strong signal. Once you have established your method, proceed with the specific HybriProbes. Please refer to Appendix A for details about the different controls.

BIOGNOSTIK's double-FITC-labelled HybriProbes are used for indirect immunogenic detection by anti-FITC-antibodies conjugated either with alkaline phosphatase (AP) or peroxidase (POD or HRP).

To establish your own *in situ* hybridization technique, please follow the steps listed below:

- First of all it is advantageous to use tissue or a cell line, known to highly express the mRNA of the gene of interest.
- It is best to start with the poly-d(T) HybriProbe directed at the poly-(A) tails to examine the mRNA quality of the investigated tissue. If fixation is incomplete, the signal will decrease in the center of the tissue. Usually, the signal is strong enough to be seen by the naked eye. In parallel, perform control experiments by using a) the HybriProbe random control and also b) without adding the probe to test if the detection system works accurately.

- Since the expression level of the gene of interest is probably unknown, use HybriProbes for the housekeeping genes beta-actin and alpha-tubulin to optimize the ISH protocol. These genes are highly expressed in virtually every cell. Please refer to Appendix A for instructions on the various controls.

Slide Preparation

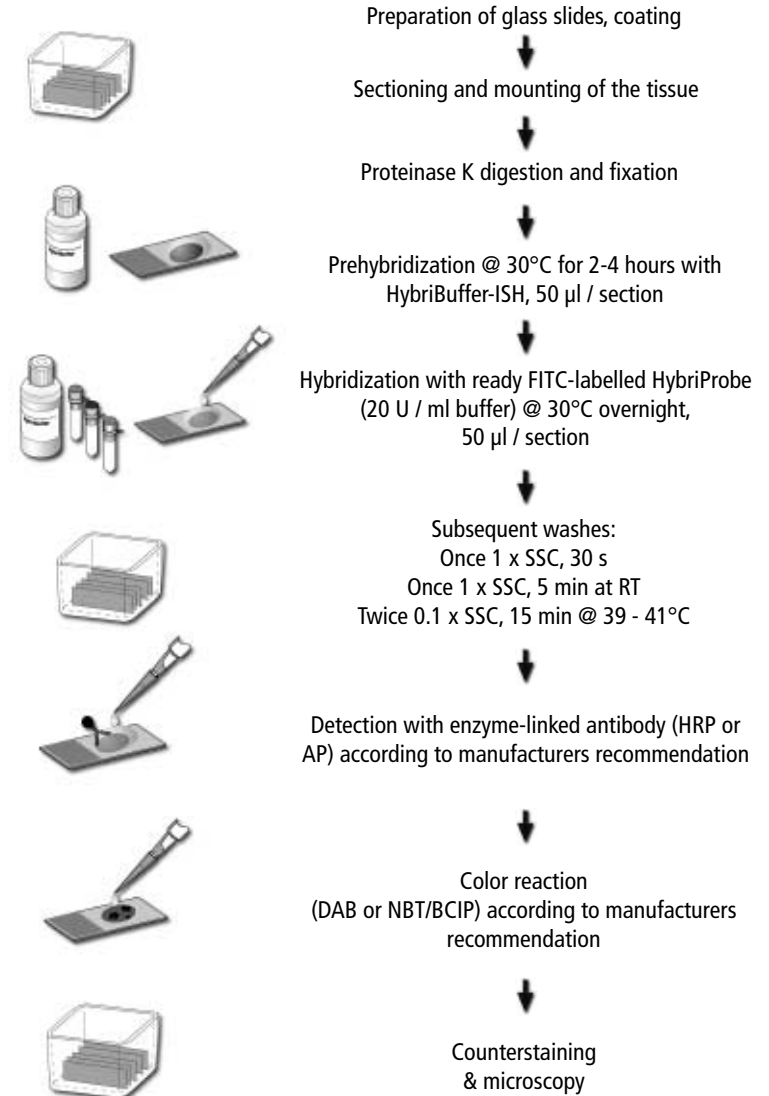
In situ hybridization is a rather harsh procedure on tissue. Slides should always be clean, fat-free and sufficiently coated to allow good adhesion of the tissue sections during incubation and washing.

- Place the slides in a tray and submerge them in warm tap water/detergent for 30 min
- Rinse thoroughly in warm tap water
- Rinse in distilled water
- Place slides into 95% ethanol/HCl (13 ml HCl per 1000 ml ethanol)
- Rinse in sterile H₂O, then dry in an oven
- Bake at 180°C for 4 h

Gelatine Coating

- **Please note:** When using Proteinase K please use silanized slides.
- Dissolve 5 g gelatine in 1 l sterile H₂O at 40°C
- Add 0.2 g of chromium potassium sulphate CrK(SO₄)₂ after gelatine has dissolved completely. (Chromium potassium sulphate is added to prevent bacterial contamination). If a high nonspecific background occurs during ISH, omit the chromium potassium sulphate.
- Dip slides into gelatine solution for 1 min
- Drain on a paper towel then dry overnight in an oven at 40°C
- If necessary repeat coating step once
- Store slides dry and dust free

Flow-Chart for Non-radioactive *In Situ* Hybridization



Silanized Slides

We recommend the gelatine-coated slides. Alternatively, silanized slides can be used but they may cause more background than gelatine-coated slides.

Tissue / Cell Preparation

Tissues

Generally, the protocols in the manual are applicable to both frozen and paraffin-embedded sections.

Since mRNA is rapidly degraded by intracellular nucleases, the tissue must either be frozen or fixed immediately after excision. This step is critical for the entire technique.

Frozen tissue sections are better because they preserve the sample morphology and mRNA quality excellently. Frozen sections may be fixed in paraformaldehyde either prior to or after freezing.

Paraffin sections can also be used, but preservation and mRNA quality may be impaired since the tissue is not preserved as quickly as with frozen sections. This may be a problem especially if the mRNA is in low abundance. Paraffin sections are routinely fixed with formalin or paraformaldehyde. Formalin fixation leads to covalent links between mRNA and proteins if kept for more than 24 h in the solution. The following recommendations should be modified according to the tissue being examined. Generally each tissue requires its own procedure with regard to the type as well as general handling in the lab. To start with, stick to this protocol unless you established a different procedure for *in situ* hybridization already.

Frozen Sections

- Immediately after removal embed tissue in mounting medium (e.g. O.C.T. compound, Miles Scientific) and freeze in liquid nitrogen.
- Cut 6-12 μM sections and mount on gelatine coated glass slides (For a higher signal cut sections thicker than 10 μM , for better localization cut them thinner).

- Keep at room temperature for 2 - 5 min, and then freeze at -20°C (Initially most conveniently in the cryostat).
- Alternatively store on a stove at $50 - 90^{\circ}\text{C}$ for 0.5 - 2 min to fix mRNA, then freeze.
- Store slides in a desiccated box at -20°C or proceed immediately with fixation.
- Fix slides in 4% paraformaldehyde/PBS for 5 min (range: 4-7 min). Then wash 2x 1 min in PBS (Alternatively fix tissues with paraformaldehyde prior to sectioning on a cryostat. Then the fixation step can be omitted at this stage).
- Do not overfix tissue since the mRNA might become inaccessible for the HybriProbes.
- **For storage only:** Subsequently dehydrate in a graded series of ethanol (70%, 80%, 90%, 96%, 100%, 1 min each). Omit this step when directly proceeding with ISH.
- If necessary, delipidate tissues with high fat content or lipid vesicles (e.g. brain tissue) in chloroform for 5 min. This step may be omitted in the preliminary experiments for establishing the method.
- Fixed slides may be stored desiccated at -80°C or submerged in 96% ethanol at 4°C .

Paraffin Sections

- Fix and embed material in paraffin according to standard procedures
- Cut 7 μM sections and mount on coated glass slides
- Dry slides in an oven overnight at 40°C
- Dewax sections 2x 10 min with fresh xylene
- Rehydrate in a graded series of alcohol

Cells

- a) Cells can be grown directly on glass slides/cover slips
- b) Cytospin is the method of choice for cells grown in flasks or microtiter plates

- Cytospin cells on glass slides at 1000 rpm for 5 min
- Air-dry at room temperature for 60 min
Fix cytospinned cells and cells grown on glass slides/cover slips in ice-cold methanol at -20°C for 10 s
- Transfer to cold acetone (4°C) for 3x 5 s
- Air-dry cells
- Store desiccated at -20°C
- Or, alternatively, fix in 4% paraformaldehyde at room temperature

c) A third method is the direct smear

- Fix suspended cells with 3 volumes of 4% paraformaldehyde at room temperature for 1 h
- Wash cells in PBS then spot onto a clean and oil-free glass slide
- Allow to air-dry
- Dehydrate in a graded series of ethanol
- Proteinase K treatment is not required for cellular preparations

Proteinase K Digestion

Frozen tissue sections usually do not require proteinase K treatment. In many tissues, however, this treatment can increase accessibility of the mRNA complexed with proteins. There is no general rule about whether to omit this step or not. Brain sections usually do not require any proteinase K treatment while other tissues like liver should be predigested. In general, a stronger fixation calls for a higher proteinase K concentration. Avoid overfixation, which stronger interferes with mRNA detection than overdigestion with proteinase K does. Overdigestion, on the other hand, can cause disintegration of the tissue. Titrate the proteinase K concentration for each different type of tissue when starting *in situ* hybridization experiments.

The recommended initial proteinase K concentration is 20 µg/ml at 37°C for 15 min. Depending on the tissue type and the degree of fixation, the most suitable concentration for the tissue under investigation should be determined empirically by titration and can range between 10-500 µg/ml.

- Always prepare a fresh proteinase K solution
- Dilute proteinase K stock in TES (50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 10 mM NaCl)
- The optimal concentration of proteinase K depends on the degree of tissue fixation
- Initially use a concentration of 20 µg/ml. Adjust the concentration between 10-500 µg/ml to achieve optimal results
- Cover each section with 30 µl or more and incubate in a humid chamber at 37°C for 15 min
- Rinse briefly in PBS
- Fix sections a second time in 0.5-1% paraformaldehyde for 5 min
- Immerse sections in sterile H₂O for 5 min
- Air-dry for 5 min
- Immediately proceed with the prehybridization step

Prehybridization

Prehybridization is necessary to prevent background staining. Sections are equilibrated since the buffer contains the same components as the hybridization buffer except for the HybriProbe. Prehybridization and Hybridization are carried out in tightly sealable plastic boxes containing a humidifying box buffer (50% formamide / 4 x SSC). Alternatively, cover sections with silanized coverslips to prevent evaporation.

- If necessary rehydrate slides in a graded series of ethanol (100%, 96%, 90%, 80%, 70%, 1 min each)
- Add box buffer to the bottom of the box. Place slides horizontally on an inset so that no box buffer can reach them
- Surround each tissue section with a ring of rubber cement (e.g. Fixogum, Marabu) or use a silicone pen
- Heat HybriBuffer ISH to 95°C, cool down on ice to 30 - 40°C
- Add 25 µl HybriBuffer ISH per cm² tissue
- Incubate section in the closed box (humid chamber) or under a coverslip at 30°C for 2-4 h

Hybridization

- Transfer HybriBuffer-ISH into a microtube
- Heat HybriBuffer ISH to 95°C, cool down to 30-40°C
- Prepare hybridization solution: add the HybriProbe directly to the HybriBuffer ISH aliquot at a final concentration of 20 units (= 60 µl $\hat{=}$ 60 pmol) HybriProbe per 1000 µl HybriBuffer ISH. (TriSeqKit: 3x 60 µl!)
- Vortex and spin this hybridization solution before use
- Draw off the prehybridization buffer and immediately replace with the hybridization solution. (The sections must not become dry during this procedure)
- Add 25 µl hybridization solution per cm² of tissue
- Hybridize sections for 10-16 h (overnight) at 30°C (use humid chamber or silanized coverslips)
- Immediately proceed with the post-hybridization procedures

Post-Hybridization Washes

Post-hybridization washes are necessary to remove probe, which has hybridized to partially, but not to entirely homologous targets. Such mismatched hybrids are less stable than perfectly matched duplexes. Salt concentration, temperature and time of the washing steps have to be adjusted to remove mismatched probes (nonspecific background) sufficiently while taking care not to significantly affect the stability of the matched hybrids (specific signal).

- Rinse sections twice for 30 s in 1x SSC to remove most of the excess probe
- Wash sections in 1x SSC for 5 min at room temperature
- Wash sections twice in 0.1x SSC with gentle agitation at 39 - 41°C (= stringent wash) for 15 min
- Depending on the tissue and on the pretreatment of the tissue, sections may be washed less stringently at 30-37°C. Adapt temperature according to signal-to-noise ratio.
- If the signal-to-noise ratio is too low, it's best to increase the stringency of the hybridization rather than of the post-hybridization washes.

Immunodetection of FITC-labelled HybriProbes with Commercially Available Kits

Non-radioactive detection has become a routine procedure in ISH. Different kits and reagents for immunodetection have been developed. They mainly differ in the choice of antibody and color substrate. We recommend the FITC detection kits from NEN (TSA™ System), DAKO (ISH Detection System for FITC-labelled Probes), Sigma (ISH Detection Kit for FITC-labelled Probes). We prefer the NEN TSA™ System because it significantly amplifies the signal and reduces nonspecific background.

BIOGNOSTIK's double-FITC-labelled HybriProbes are used for indirect immunogenic detection by anti-FITC antibodies linked either to alkaline phosphatase (AP) or peroxidase (POD or HRP). Please follow the manufacturers recommendations when using a FITC detection kit.

Example Protocol for Signal Detection and Visualization

The following section contains a sample standard protocol for signal detection and visualization.

Initial Washing and Blocking Step

Required Buffers:

1. Washing buffer: PBS or 100 mM Tris-HCl
2. Block Buffer: Washing buffer (PBS or 100 mM Tris-HCl) + a detergent: 0.1% Tween® 20 or 0.1% Triton® X-100 + a protein-based blocker: 2% BSA (monoclonal antibodies) or 2% normal serum (polyclonal antibodies)

First, nonspecific binding sites for the antibody have to be blocked.

- Wash slides 2 x 10 min in washing buffer alone
- Incubate slides 2 x 10 min with block buffer

Antibody Incubation and Washing

Initially, it may be necessary to determine the most suitable antibody concentration. Incubate several sections with different antibody dilutions. If the manufacturer recommends 1:500, also try 1:1000 and 1:100.

- Add the antibody to the block buffer, pipette this solution to the sections
- Incubate sections for 2 h in a humid chamber
- Wash sections 2x 10 min in washing buffer

Visualization of the Signal

We recommend to use the commercially available FITC detection kits as described above.

Alkaline phosphatase (AP) in conjunction with NBT/BCIP color substrates is described here because of its popularity. The POD/DAB system produces similarly good results. The procedures are basically similar to the AP/NBT/BCIP system.

The anti-FITC antibody is most commonly linked to alkaline phosphatase that works at a neutral pH, although its optimum is at pH 9.5. Alkaline phosphatase turns NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) into insoluble NBT formazan (nitroblue tetrazolium formazan). NBT formazan is dark blue and has reflective properties.

Both NBT and BCIP are available as ready-to-use solutions or tablets (Roche Diagnostics, Sigma). Alternatively, prepare the solution with 0.18 mg/ml BCIP, 0.34 mg/ml NBT, 240 µg/ml Levamisole (blocks endogenous alkaline phosphatases) in alkaline detection buffer (see below).

Levamisole blocks the endogenous alkaline phosphatases found in some tissue types that can cause nonspecific signals. The alkaline phosphatase bound to the detection antibody is an isoenzyme of pancreatic origin that is not blocked by Levamisole. If you choose to work without Levamisole, perform a control experiment first by incubating a tissue slide with the detection reagent without adding the antibody linked to alkaline phosphatase. If the section does not produce a signal after incubation, endogenous alkaline phosphatase activity can be ruled out.

Protocol:

- Prepare the alkaline detection buffer: 100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂. Adjust to pH 9.5, always prepare freshly
- Prepare NBT/BCIP solution according to the manufacturers instructions.
- Incubate sections 10 min in alkaline detection buffer
- Cover each section with 200 µl of NBT/BCIP solution containing Levamisole.
- Incubate slides for 2 - 24 h in a humid chamber in the dark. Slides may be submerged and kept in a standing position to avoid nonspecific NBT formazan precipitation on the sections
- Monitor the color reaction under a microscope. Avoid prolonged exposure to light during the reaction
- Stop reaction with stop solution (10 mM Tris-HCl, 1 mM EDTA, pH 8) once the staining is optimal
- Dip slides in distilled water

Counterstaining and Microscopy

- Counterstain tissue/cells according to your established protocols
- Mount sections in a water-based medium (e.g. Aqua Mount, Lerner Lab., Pittsburgh), since xylene-based media may destroy the precipitate
- Brightfield microscopy is the most relevant and routinely used procedure

Optimizing and Troubleshooting

If the results of the procedure are not satisfactory, try to locate the cause.

- Tissue preparation and fixation affect RNA quality
- Fixation and proteinase K digestion affect RNA accessibility
- Hybridization and post-hybridization affect hybrid stability and hybrid specificity
- Non-radioactive detection may be optimized when necessary

Checking the Detection System and Labelling Efficiency

It is important to check the quality of the detection system and the labelling efficiency of the FITC-labelled probes when problems with the signal detection occur. The labelling efficiency and performance of the detection system can be checked on a nylon membrane, which is suitable for blot hybridizations. Perform a Dot Blot experiment as described below and use the buffers and antibodies as recommended by the manufacturer of the detection kit.

Dot Blot:

- Dilute 1 μ l (1 pmol) of the HybriProbe in 9 μ l of H₂O
- Spot 1 μ l of this dilution (1:10) on a nylon membrane
- Optionally spot further dilutions (1:2; 1:5; 1:50)
- Allow the membrane to dry for 20 min
- Bake membrane at 80°C for 30 min or cross-link with UV-light
- Perform the detection according to the instructions of kit manufacturer.

The usual steps are:

- Wash membrane with washing buffer
- Place membrane in blocking solution for 30 min at RT
- Incubate membrane with diluted antibody solution for 30 min at RT
- Wash membrane twice with washing buffer
- Proceed with immunogenic detection as recommended by the manufacturer of the detection kit

High Background

1. Increase the hybridization temperature by 5°C. In a second series, increase the stringent post-hybridization wash temperature in a first step by 5°C.
2. Use the random Control HybriProbe (see Appendix A). If background is a problem, this can probably not be overcome by increasing the hybridization temperature. More likely, the problem involves tissue preparation or the detection steps.
3. When using non-radioactive detection systems, a high background may be due to insufficient washing steps or problems related to the detection system.

Modify washing steps (increase time and temperature) and the concentration of the blocking reagent as recommended by the supplier of the detection reagents/kit.

Check if high background is related to unspecific antibody binding during detection:

Omit adding FITC-labelled probe during hybridization step, no signal should occur, otherwise something is wrong with the detection system.

When using the AP/NBT/BCIP system check if high background is related to endogenous enzymatic activity of the fixed tissue: Process sections as usual but omit the hybridization step and omit adding the FITC-antibody. Staining indicates activity of cellular alkaline phosphatase. In such case add Levamisole as described above in section „Visualization of the Signal“.

4. Depending on availability use different antibodies since some can produce nonspecific signals.
5. If you cannot rule out whether the signal is specific or not, digest RNA with RNase prior to hybridization. There should be no signal after this step. Be careful not to cross-contaminate routine equipment with RNase. This may interfere with subsequent hybridizations.
6. Background may be intracellular or even extracellular. Extracellular background may be due to precipitation of the antibody or signal enhancers like tyramide. In this case, submerge the slides upright in the detection solution to avoid nonspecific precipitation on the slide.

No Signal

Different steps may have to be tested or modified:

1. Perform a technical control by detecting the expression of the housekeeping genes beta-actin and alpha-tubulin or the poly-d(A) tails. (See Appendix A). A general problem with RNA detection is rapid RNA degradation by nucleases. Make sure that you establish the procedure with tissue containing intact RNA. The HybriProbes for poly d(T) and the housekeeping genes beta-actin

and alpha-tubulin can also be used to verify the integrity of the RNA. Bake all glass ware, treat water with DEPC (but not Tris-buffer), and use gloves.

2. Housekeeping genes can also be used to establish the optimal balance between fixation and proteinase K digestion. Perform parallel series in one or more experiments to determine the optimal conditions for each tissue used.
3. Use a cell type with known high expression of the gene (positive control).
4. The concentration of the labelled probe may be too low. Double the concentration of the labelled probe in the hybridization solution.
5. The hybridization step may be too stringent: Decrease the hybridization temperature by 5°C. If nonspecific noise is not a problem, the hybridization temperature can be lowered incrementally, even down to room temperature. In a parallel series but not while lowering the hybridization temperature, decrease the temperature of the stringent washing step. Sometimes room temperature will be sufficient also for the washing step but this again depends on the noise which will increase gradually.
6. Detection step: It may be necessary to increase the antibody concentration or to increase the incubation time.
7. Add 10% polyvinyl alcohol (70–100 kDa, Sigma) to the detection solution (e.g. to NBT/BCIP solution) to increase the signal.
8. If your gene is only weakly expressed, intensify the signal. Two options should be considered:
 - Use the HybriProbe Custom DesignTriSeq Kits with 3 HybriProbes directed at different regions of the targeted mRNA
 - Use NEN's tyramide-based enhancer kit (TSA™ System) that increases the signal 100-1000 fold. NEN's fluorescein-based tyramide kit is compatible with *BIOGNOSTIK*'s HybriProbes (see Appendix C)

Appendix A

Choice of Controls for ISH

Control experiments with both positive and negative controls are very important for validating the specificity of the detection signal. The interpretation and reliability of results critically depend on control experiments performed under comparable conditions. This is why *BIOGNOSTIK* combines specific HybriProbes and controls in each kit along with an optimized buffer system. To establish the *in situ* hybridization technique, we recommend starting with the technical control and tissue control both of which should give a strong signal. Once the method is well established, proceed with the specific HybriProbes.

Positive Controls:

Use a tissue or cell line known to contain the mRNA of interest in sufficient amounts. If possible or necessary, stimulate gene expression prior to detection.

Negative Controls:

1. Negative sample: Use a tissue or cell line which is known to lack the mRNA of interest.
2. Target digestion: Tissue mRNA may be digested enzymatically with RNase prior to ISH. This test should only be carried out if a separate room and separate glassware can be used. RNase is very stable and impedes ISH if the same glassware is used in routine procedures.
3. Random control: All of *BIOGNOSTIK*'s random control HybriProbes are designed to lack relevant homologies to other mRNA sequences. The result of this control should also be a blank slide.
4. No probe: Omit the probe to test if the detection system shows nonspecific results. Background staining should be very low. If a signal occurs, the antibody might have bound nonspecifically or endogenous alkaline phosphatase activity (NBT/BCIP-System) is responsible for the signal.
5. No probe + no antibody: If a signal showed up without adding the probe (see experiment 4) and you are working with the AP/NBT/BCIP system, check if high background is related to endogenous enzymatic activity of the fixed tissue: Omit the hybridization step and omit adding the FITC-antibody. Staining of the sections indicates activity of cellular alkaline phosphatase. In such

case add Levamisole as described above in section „Visualization of the Signal“.

Technical and Tissue Control:

The poly-d(T) HybriProbe should give a strong and ubiquitous intracellular signal since most mRNAs contain a poly-d(A) tail. The signal is often visible to the naked eye. Even though a positive signal validates the technique, it does not necessarily mean that the mRNA is fully intact. The poly-d(T) HybriProbe is not 100% proof for intact mRNA since it can hybridize to any part of the poly-d(A) tail. Partially degraded mRNA might still provide enough poly-d(A) fragments that are large enough for hybridizing to the poly-d(T) HybriProbe. Therefore, this probe should be used first to validate the technique. In a second step, use the tissue controls described below to validate the quality and integrity of the mRNA.

A positive signal by the Poly-d(T)-HybriProbe together with a negative signal by the random control-HybriProbe indicates that the tissue is well prepared, i.e. the mRNA is accessible. Now, the next question is: is the mRNA still intact?

mRNA and Tissue Control:

To validate the quality and integrity of the mRNA, use the HybriProbes for mRNA of the abundant housekeeping genes beta-actin and alpha-tubulin. If these tissue control HybriProbes give a positive signal while the random control is negative, your system is working well and the mRNA is of good quality. The control HybriProbes are also useful for semiquantitative assessments of the signals when used concurrently with specific HybriProbes.

Appendix B

Solutions

20x SSC is a stock solution containing 3 M NaCl and 0.3 M sodium citrate

350 g	NaCl
166 g	sodium citrate (Trisodium Salt: Dihydrate)
1700 ml	autoclaved double distilled water
2000 ml	end volume

- Adjust pH to 7.0 with NaOH or HCl.

4% paraformaldehyde / PBS, 100 ml (always prepare freshly)

- Weigh 4 g of powdered paraformaldehyde into a glass bottle
- Add 60 ml PBS.
- Heat to max. 60°C, stir continuously until paraformaldehyde is dissolved completely.
- Pour through a Whatman-type paper filter.
- Add PBS to 100 ml.
- Do not autoclave, no pH adjustment is necessary.

Appendix C

Recommendation for Non-Radioactive FITC-Detection Kits

Dako (www.dakocytomation.com)

GenPoint™ Fluorescein Tyramide Signal Amplification System for in situ hybridization is a highly sensitive and convenient in situ detection system for use with fluorescein-labeled probes. The peroxidase-tyramide amplification chemistry is optimized to deliver excellent sensitivity and signal localization. The ready-to-use reagents provide rapid, reliable results, and are qualified for mRNA and DNA ISH applications. The amplified fluorescein signals may be viewed either as fluorescence, or converted to chromogen for permanent viewing under bright-field illumination. Components included: TBST wash buffer, hydrogen peroxide blocking reagent, primary anti-FITC-HRP reagent concentrate, anti-FITC-HRP diluent, fluorescyl tyramide, secondary anti-FITC-HRP reagent, DAB chromogen, and DAB chromogen buffer.

Sigma (www.sigma-aldrich.com)

ISH Detection Kit for FITC-labelled Probes