

*Roche Applied Science*  
**Nonradioactive**  
***In Situ* Hybridization**  
**Frequently Asked Questions**



**The DIG System –**  
*Highly Sensitive. Short Exposure Times. Safe Handling.*

# General Overview

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Roche Applied Science was one of the first companies to offer a nonradioactive technology allowing customers to move away from the use of hazardous radioactive isotopes. Even after 16 years, the DIG System remains **the** nonradioactive technology of choice to label and detect nucleic acids for multiple high sensitive applications such as *in situ* hybridization or filter hybridization.

Compared to radioactive labeling and detection techniques, the DIG System has multiple advantages:

- High sensitivity (more sensitive than radioactive methods)
- Precise localization of hybridization signals
- Time saving: Short exposure times (minutes rather than hours or days)
- Safety (no contact with hazardous materials - no environmental contamination)
- Reproducibility (Probes are stable for a minimum of one year and thus are reusable repeatedly)
- Well-established protocols (many years of experience)

The DIG System allows the safe and efficient labeling of DNA and RNA, or oligonucleotide probes. These probes can be used for all many different applications.

Roche Applied Science provides a wide variety of kits and individual reagents for the labeling and detection of nucleic acids by different methods. A detailed overview can be found in the **DIG Product Selection Guide** or on our dedicated website at <http://www.roche-applied-science.com>.

For detailed information on products and protocols we recommend for further reading:

**DIG Product Selection Guide**

**Nonradioactive *In Situ* Hybridization Manual**

**DIG System *In Situ* Hybridization Reference List**

**DIG Application Manual for Filter Hybridization**

**Lab FAQs**

**DIG Special Interest Site**

*All these brochures and manuals can be downloaded from our website*

<http://www.roche-applied-science.com>.

*For hardcopies please contact your local Roche Applied Science supplier.*

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# 1. General *In Situ* Hybridization (ISH)

## Overall Sensitivity of Nonradioactive ISH

### Question

How sensitive is nonradioactive *in situ* hybridization compared to radioactive ISH?

### Answer

A rapidly increasing number of researchers are using the DIG system for their *in situ* hybridization applications. This is because the DIG system shows sensitive and specific results in a very short detection time. Exposure times necessary for radioactive experiments can vary from days, weeks and sometimes even months have to be accepted! Incubation times take minutes to hours (in extreme cases overnight) when using the DIG system (e.g., **Anti-Digoxigenin-AP**, **Fab fragments**) and colorimetric detection with **NBT/BCIP**. Another advantage of nonradioactive detection is that the color precipitate can be more precisely correlated with cells expressing the gene of interest than is possible with the signals generated by radioactivity.

For selection of the appropriate kits and labeling mixes please refer to the **DIG Product Selection Guide**.

**Anti-Digoxigenin-AP, Fab fragments**  
**NBT/BCIP**

For some applications where fluorescence detection is preferred our **Fluorescent Antibody Enhancer Set for DIG Detection** can further enhance sensitivity if necessary.

## Detection Limits using Nonradioactively labeled Probes

### Question

Could you give us the information about the detection limit (e.g., copy number) when using various hapten- (DIG-, Biotin-, fluorescence-) labeled nucleic acid probes for *in situ* applications, especially rare mRNA detection?

### Answer

► It's difficult to give a concrete value of the detection limit (e.g., copy number – because it also depends on the type of expression pattern). For example, rare transcripts which are transcribed in different tissue types will be harder to locate than transcripts which are specifically transcribed in distinct cell types. In general, with total or mRNA, Northern experiments are performed to check the probe and the transcription pattern in different tissue preparations prior to *in situ* hybridization. If you receive a signal in the Northern blot analysis – try out *in situ* hybridization and include positive<sup>1</sup> and negative<sup>2</sup> controls. Thus for rare mRNA detection please use RNA probes!!!

<sup>1</sup>A gene known to be transcribed in a distinct pattern in the tissue type / or surrounding the tissue of interest

<sup>2</sup> Sense control / no probe at all / competition with unlabeled antisense probe

► Which system is recommended – **DIG, Biotin or fluorescence**?

**DIG:** For detection of rare transcripts in tissues use the DIG / AP system with **NBT/BCIP** for detection. References and protocols – please refer to our **Nonradioactive *In Situ* Hybridization Manual**.

# 1. General *In Situ* Hybridization (ISH)

**Biotin:** In general, all our recommendations for the DIG system are also true for the Biotin system. We strongly promote the use of DIG label in favour of the Biotin label for the following reason: Biotin is a lot more “sticky” than DIG:

▶ Biotin occurs endogenously in almost every tissue and cell. When working with unpurified DNA or in tissues and cells, unspecific side reactions can occur. Streptavidin, which is used primarily for the detection of Biotin, tends to stick non-specifically to all solid supports like microplates and membranes, causing background.

**Fluorescence:** Protocols (e.g., for chromosome spreads) are published in the [Non-radioactive \*In Situ\* Hybridization Manual](#).

In general, the main drawbacks of the using fluorescence in combination with tissue include strong autofluorescence of the tissue, or background fluorescence, rapid fading of signal due to wrong choice of mounting media: appropriate anti-fading mounting media should be used.

For some applications where fluorescence detection is required use the [Fluorescent Antibody Enhancer Set for DIG Detection](#) to further enhance sensitivity.

## Alkaline Phosphatase in Comparison to Peroxidase System

### Question

I have a question concerning nonradioactive ISH ([DIG RNA Labeling Kit](#) and [DIG Nucleic Acid Detection Kit](#)). I used to work with [Anti-DIG-AP](#) for the detection of [cRNA probes labeled with DIG](#) and it works fine using [NBT/BCIP](#) as an enzymatic substrate.

Recently I tried to switch to [Anti-DIG-POD system](#) and it does not work. Do you have any suggestions or comments? I would like to use this system because detection with [DAB substrate](#), would be more convenient for my work.

### Answer

Generally, the [Anti-DIG-AP / NBT/BCIP](#) system is more sensitive than the [POD / DAB](#) system. Problems associated with extremely high background are often encountered when switching from AP the POD for ISH. Skip the [Proteinase K](#) and HCl steps during the pretreatment steps. However, and if these steps are required, perform a second fixation step (e.g., in 3 – 4% Paraformaldehyd/PBS) after this treatment.

## Choosing the Right Labeling Method for Your Experiment

### Question

Can you give us tips on which labeling methods to choose and which accompanying products are generally available?

### Answer

Please refer to our [Nonradioactive \*In Situ\* Hybridization Manual, p. 15 – 16, Choosing the Right Labeling Method for your Hybridization Experiment](#).

For selection of the appropriate kits and products please refer to the [DIG Product Selection Guide](#).

# 1. General *In Situ* Hybridization (ISH)

## Choice of Probe / Probe Spanning Region of cDNA

### Question

Is it better to use the whole cDNA as a probe, or to select a region of the gene as a probe?

### Answer

If you use RNA as a probe, we suggest using a subregion of the gene as a probe. In most cases the 3'-UTR, and regions close to it are most suitable, because they are often unique to the gene. It is important that the region you use as a probe does not bear any homology to other genes. The probe should be at least 300 base pairs long, the optimal length is 600-1000 base pairs. For cloning of appropriate fragments use the [Rapid DNA Ligation Kit](#) and for subsequent restriction digestion please refer to our catalog section restriction enzymes or to [Restriction Enzymes FAQs and Ordering Guide](#). For labeling of RNA probes with DIG use the [DIG RNA Labeling Kit](#). For selection of the appropriate kits and labeling mixes please refer to the [DIG Product Selection Guide](#).

## General Protocol for DIG-labeled RNA Probes and Tissue Sections

### Question

Do you have a protocol or kit for *in situ* hybridization (tissue sections) using DIG-labeled RNA probes?

### Answer

We offer many kits and components for *in situ* hybridization. Some examples include the [DIG RNA Labeling Kit](#) and the [DIG Nucleic Acid Detection Kit](#), which are used together for *in situ* hybridizations. For more detailed information and protocols, please view our [DIG Product Selection Guide](#) and check out our [Nonradioactive In Situ Hybridization Manual](#) for detailed protocols for your specific experimental requirements.

## High Throughput ISH

### Question

Do you have a protocol or advice for high throughput *in situ* hybridization?

### Answer

Gene expression analysis is the key procedure in determining the function of genes and the activities of promoters. With the completion of many genome projects a large number of gene sequences are readily available for comprehensive functional analysis. In contrast to microarrays and other gene expression technologies, *in situ* hybridization (e.g., using DIG-labeled RNA probes) is a non-destructive RNA localization technique that provides the highest level of cellular resolution and information. However, *in situ* hybridization is a slow, labor-intensive and error-prone procedure when carried out manually.

# 1. General *In Situ* Hybridization (ISH)

Automation of nonradioactive *in situ* hybridization overcomes these disadvantages by exerting accurate control of critical parameters such as temperature, pipetting volume, incubation time and numbers of repetitions. Throughput can easily be adjusted to 200 slides per week or more.

Different instruments for automated nonradioactive *in situ* hybridization are now on the market – optimised for different applications such as whole-mount or tissue section *in situ* hybridization; some of these are also suitable for immunohistochemistry applications. The adaptation of nonradioactive *in situ* hybridization for high-throughput and automation largely follows the manual procedure and relies on the same high-quality, specific and sensitive detection reagents.

Reference:

Hantke, S. (2004) The DIG System – Nonradioactive Automated High-Throughput *in situ* Hybridization: a powerful tool for functional genomics research. **Biochemica No. 1, 18 – 20.**

## 2. Fluorescence *In Situ* Hybridization (FISH)

### Direct (FITC-UTP) versus Indirect (Anti-DIG-FITC) Labeling System

#### Question

For *in situ* hybridization of frozen sections, I would like to use a FITC system. Which has higher sensitivity – the direct (FITC-UTP) or indirect (Anti-DIG-FITC) labeling system?

#### Answer

Indirect (Anti-DIG-FITC) labeling systems are always superior to direct (FITC-UTP) in terms of sensitivity. It largely really depends on how abundant your target is and on the type of tissue – since fluorescence detection might lead to background due (tissue auto-fluorescence). Preadsorption of the antibody may help minimize background (see details in the [Nonradioactive \*In Situ\* Hybridization Manual](#)). Have you ever thought of trying Anti-DIG-AP / NBT/BCIP color detection system? If you need to stick to fluorescence we recommend our [DIG-Nick Translation Mix](#) in combination with the [Fluorescent Antibody Enhancer Set for DIG Detection](#) for high sensitivity applications.

### Sensitivity FISH / Enhancements via Cascades

#### Question

What is the difference in the sensitivity of DIG detection between [Anti-DIG-Fluorescein](#) and [Fluorescent Antibody Enhancer Set for DIG Detection](#)?

#### Answer

Refer to the package insert linked to our website for all the details on the [Fluorescent Antibody Enhancer Set for DIG Detection](#) (includes 1 primary and 2 secondary antibodies for amplifying a fluorescein signal when detecting DIG on a glass slides under a fluorescent microscope).

The sensitivity of course should be higher using an antibody cascade – but the overall sensitivity is also dependent on the application, the labeling efficiency of the probe and the experimental conditions. If you are planning nonradioactive *in situ* hybridization please refer to the protocols in the [Nonradioactive \*In Situ\* Hybridization Manual](#).

### Protocol for Chromosome Spreads

#### Question

Do you have a protocol for chromosome spreads and DNA probes labeled via nick translation?

#### Answer

For details on procedures for *in situ* hybridization to chromosomes, cells, and tissue sections *e.g.*, multicolor FISH please refer to Chapter 5 of the [Nonradioactive \*In Situ\* Hybridization Manual, p. 78f.](#)

Further reference:

**Protocol:** Detection of human chromosomes in metaphase spreads using DIG- or Fluorescein-labeled nick translated human satellite probes can be found under the following [link](#):

[http://www.roche-applied-science.com/ots/pdf/protocol\\_chromosome.pdf](http://www.roche-applied-science.com/ots/pdf/protocol_chromosome.pdf)

## 2. Fluorescence *In Situ* Hybridization (FISH)

### Recommendations for FISH Probes: Nick Translation Mixes

#### Question

Which are the benefits of premixed Nick Translation Mixes *e.g.*, **DIG-Nick Translation Mix**; **Biotin-Nick Translation Mix**; **Nick Translation Mix**? Which further recommendations and troubleshooting information can you supply?

#### Answer

Probes prepared with the **DIG-Nick Translation Mix** for *in situ* probes are especially qualified for *in situ* hybridization applications (*e.g.*, FISH on chromosome spreads) but can also be used for filter hybridization techniques. Note: For highly sensitive filter hybridization probes use **DIG-High Prime**. For nonradioactive labeling of *in situ* probes with other haptens and fluorophores Roche Applied Science offers the **Biotin-Nick Translation Mix** and the **Nick Translation Mix** (without labeled nucleotides, therefore labeling with your hapten of choice is possible).

#### *Ideal Fragment Length:*

In *in situ* hybridization experiments, the fragment length distribution severely influences the efficiency of hybridization. The use of probes showing fragment lengths above the optimal range of 200 – 500 nucleotides usually results in enhanced spotty background signals due to unspecific sticking of the probe to *e.g.*, the glass surface. It also can result in reduced accessibility to the target nucleic acid (like metaphase chromosomes or cellular and tissue targets). On the other hand use of probes being excessively short will result in poor hybridization efficiency and sensitivity. This is due to fast rehybridization kinetics of short fragments yielding a high proportion of “snapback” probe DNA that reduces the probe amounts for hybridization to the target. Thus, the level of DNase I is of high relevance in probe labeling for *in situ* applications. The use of the premixed nick solution reduces pipetting steps and increases the reproducibility of the labeling reaction.

#### *Sample Material*

- Supercoiled and linearized plasmid DNA
- Supercoiled and linearized cosmid DNA
- Purified PCR products
- Genomic DNA

Note: Denaturation of the template before nick translation is not required.

#### *Troubleshooting Recommendations:*

Efficiency / No Signals with FISH

- ▶ If your FISH shows no signals, check probe length; if probe length is less than 200 – 500 bp but around 50 bp, check if enough template (minimum 1 µg) was used in labeling. Follow the protocol mentioned in the **package insert** of the respective kit (*e.g.*, **DIG-Nick Translation Mix**) closely.
- ▶ Use aliquots (2 – 3 portions) of the Nick Translation Mix solution because repeated freezing and thawing destroys the mixes!
- ▶ Our translation mixes are very sensitive to the DNA concentration that is adjusted

## 2. Fluorescence *In Situ* Hybridization (FISH)

- ▶ Our translation mixes are very sensitive to the DNA concentration that is adjusted in the labeling reaction. The variation of template amount can lead to labeled fragments of increased size distribution. Therefore it is important to carefully determine the template concentration and to follow the recommendation in the **package insert** of the **DIG-Nick Translation Mix**. The labeling volume should be 20 µl for each µg of template.

### HNPP\*/ Fast Red (AP substrate) for FISH

#### Question

For which applications do you recommend the **HNPP Fluorescent Detection Set**. Which further recommendations and troubleshooting information can you supply?

\*2-Hydroxy-3-naphthoic acid-2'-phenyl- anilide phosphate

#### Answer

In combination with Fast Red, HNPP is used for sensitive fluorescent detection of non-radioactively labeled nucleic acids in fluorescence *in situ* hybridization (FISH) and membrane hybridization. HNPP/Fast Red gives a bright, long-lasting red or orange signal withstanding long exposures to UV-light.

HNPP is a fluorescent substrate for alkaline phosphatase that enables sensitive detection of biomolecules. Upon dephosphorylation, HNPP produces a strongly fluorescent precipitate. For *in situ* applications HNPP is coupled with the diazonium salt Fast Red TR to enhance the affinity to tissues and chromosome preparations and to ensure precipitation of the dephosphorylated form (HNP) *in situ*. The reaction product generated after dephosphorylation (HNP/Fast Red TR) is a highly fluorescent precipitate that accumulates during the reaction. The accumulation can be triggered by repeated addition of fresh substrate solution. The absorbance maximum of combined HNP/Fast Red TR precipitate is shifted towards 553 nm and a broad fluorescence emission (between 565 – 620 nm) with a maximum at 584 nm is generated.

For details and the protocol please refer to the package insert of the **HNPP Fluorescent Detection Set**.

Reference: Sagner, G: DNA *in situ* hybridization with an alkaline phosphatase-based fluorescent detection system **Nonradioactive *In Situ* Hybridization Manual, p. 124f.**

## 2. Fluorescence *In Situ* Hybridization (FISH)

### Double FISH / Multicolor FISH

#### Question

I would like to detect simultaneously different targets in chromosome or in tissue. Can you please supply me with protocols to visualize the signals with DIG-, Biotin-, or fluorochrome-labeled probes?

#### Answer

Please check out the protocols in our [Nonradioactive \*In Situ\* Hybridization Manual, p. 78f, Chapter 5: Multicolor FISH and procedures.](#)

Further references:

Hughes, S.C.; Krause, H.M. (1998) Double labeling with fluorescence *in situ* hybridization in *Drosophila* whole-mount embryos. *BioTechniques* 24, 530 – 532.

Saunders, C., Cohen, R.S. (1999) Double FISH and FISH fluorescence immunolocalization procedures for whole-mount *Drosophila* ovaries. *BioTechniques* 26, 186 – 188.

The references and web resources used during the development of this protocol, and of general relevance to the subject of fluorescent *in situ* hybridization are available under: <http://www-biology.ucsd.edu/~davek/inforefs.html>

### FISH: Weak or Missing Signals

#### Question

There is no signal or only a very weak fluorescence signal detected. How can we improve the results?

#### Answer

Missing or very weak fluorescence signals are obtained due to different reasons. Since the FISH procedure includes a considerable number of critical steps, check all the steps carefully, in order to identify the potential problem.

#### *Insufficient Labeling of the Probe*

This is one of the most common causes for weak signals. In most cases, DNA-probe labeling is the problem. Depending on the source and purity of the DNA probe and the labeling method, several contaminants may interfere with the *in vitro* labeling procedure.

- ▶ Check the labeling efficiency of the probe by dot blot analysis. Refer to the detailed protocols in the [DIG Application Manual for Filter Hybridization, p. 16, Chapter 1: Evaluation of Probe Labeling Efficiency](#) or [p. 79ff, Chapter 2: Estimation of Probe Yield by the Direct Detection Procedure.](#)
- ▶ It is important to use defined amounts for DNA labeling (refer to the respective package insert of the labeling kits). In particular, too high amounts of DNA lead to a lower incorporation of labeled nucleotides per base pair which corresponds to a less intense fluorescence signal. It is also always advisable, to have a positive control (a labeled probe which has been shown already to give routinely good results in FISH experiments).

## 2. Fluorescence *In Situ* Hybridization (FISH)

### *Probe Size*

One should keep in mind, that the fluorescence signal is proportional to the number of DIG-labeled bases per probe molecule. This is especially relevant in FISH experiments in which single copy sequences in non-polytene chromosomes shall be hybridized, but also in polytene chromosomes very short probe molecules can lead to weak fluorescence signals. Experience showed that the minimal length for detecting a probe in polytene chromosomes is approximately 350 base pairs.

There are several possibilities to enhance the sensitivity. One easy way is to ligate a short probe to itself prior to labeling, thereby artificially lengthening the molecules. A second approach of enhancing the hybridization signal is to include 10% dextran sulfate in the hybridization buffer; this results in a significant increase in sensitivity by promoting “branching” effects during hybridization. However, the dextran sulfate enhancement may result in a significant increase in background fluorescence. Sometimes a strong hybridization signal with a high background is more difficult to interpret than a weak fluorescence signal with little or no background fluorescence. For more information, refer to the package inserts of the **DIG-Nick Translation Mix** in combination with the **Fluorescent Antibody Enhancer Set for DIG Detection**.

### *Quality of Chromosome Preparations*

One frequent reason for failure of FISH is the quality of the chromosome preparation. For good or very good hybridization results, chromosome preparations with intact DNA in the chromosome is a prerequisite for good hybridization results. There are several ways to loose DNA during the preparation of chromosomes. Any contact with potential DNases should be avoided (handling of the slides with gloves is recommended). The chromosomes have to be stabilized by fixation (usually done by treatment with 40% v/v acetic acid, also 4 – 6% formaldehyde fixation is commonly used). The chromosome preparations should be stored in 100% ethanol or isopropanol at –20° C. This prevents loss of DNA by the action of endogenous nucleases as well as bacterial growth and digestion of DNA in chromosomes by bacteria. An additional positive effect of the prolonged storage in ethanol or isopropanol is a “hardening effect” which stabilizes the chromosome structure during the rather harsh treatments of the chromosomes, which are necessary to denature the chromosomal DNA.

For more information, refer to the protocol attached to this [link](#) or refer to the **Nonradioactive *In Situ* Hybridization Manual, p. 108f, A simplified and efficient protocol for nonradioactive *in situ* hybridization to polytene chromosomes with a DIG-labeled DNA probe.**

### *Insufficient Denaturation*

To obtain a good hybridization result it is necessary to denature the chromosomal DNA. The denaturation is usually performed by treatment with 0.7N NaOH. This denaturation procedure gives very good and reproducible results. In some cases, however, other denaturation methods might be more suitable (heating to 100°C, exonuclease digestion, treatment with HCl etc). Depending on the type of chromosomes, sometimes one or the other procedure might either not be sufficient to denature the DNA in the chromatin completely or, which is especially observed after boiling the chromosomes, the DNA is extracted from the chromosomes (refer to the FAQ **“Strong Diffuse Fluorescence Signals Next to the Chromosomes”**).

## 2. Fluorescence *In Situ* Hybridization (FISH)

### Chromosome Preparation Shows no Fluorescence

#### Question

The chromosome preparation looks like covered with a thin film and no fluorescence is visible.

#### Answer

Sometimes lipids may be present during preparation which can dissolve in ethanol or isopropanol. When the slides are removed from the alcohol solution and air dried, the lipids form a thin film over the entire preparation with a dramatic effect on the FISH result. The lipid film prevents the contact of all probes in aquatic solvents to the chromosome preparation, with no chance to get a positive result. This also happens, when the chromosome preparations are handled without gloves, in particular when fatty fingers are submersed into the ethanol/isopropanol in which the chromosome preparations are stored.

### High Background Fluorescence / Recommendations to Reduce Background

#### Question

The hybridization result (fluorescence signal) is visible in a sufficient intensity but there is a high background fluorescence which obscures the result.

#### Answer

The reason is possibly unspecific sticking of the labeled DNA to proteins or other DNA-binding molecules in the tissue.

- ▶ Include **Blocking Reagent** to your detection procedure. For general recommendations concerning blocking methods please also refer to the **Nonradioactive *In Situ* Hybridization Manual, p. 25, Chapter 2: Details of the Technique**. Our collaborators discovered that 0.1 to 0.5% SDS in the hybridization buffer will help to reduce the background significantly. If this does not help, one can “preincubate” the slide with an hybridization buffer containing up to 100 ug/ml of a mixture of single stranded and double stranded carrier DNA for two hours under hybridization conditions. The ssDNA is most effective to reduce the unspecific background binding. It works like a blocking reaction. The same carrier DNA should also be included in the real hybridization mixture but unfortunately this treatment sometimes also reduces the strength of the hybridization signal, however the ratio of signal to background is much better in any case.
- ▶ A more drastic way to reduce background fluorescence is the treatment with 0.25% acetic anhydrid in triethanolamine. (Hayashi, S., Gillam, I.C., Delaney, A.D., and Tener, G.M. (1978) Acetylation of chromosome squashes of *Drosophila melanogaster* decreases the background: J. Histochem. Cytochem. 26: 677 – 679. This treatment results in acetylation of proteins thereby causing a chemical blocking of binding sites.

## 2. Fluorescence *In Situ* Hybridization (FISH)

### Unexpected Multiple Fluorescent Signals

#### Question

Although only one chromosomal locus is expected, multiple fluorescence signals are detected in many chromosomal locations. What can be the cause of this result?

#### Answer

The hybridization probe may contain (highly) repetitive elements or transposons. Make sure that the probe is free of repetitive elements. An appropriate test is to use the probe for a Southern analysis versus genomic DNA of the organism concerned. If there is a smear or many hybridizing bands are detected, this indicates the presence of repetitive elements in the probe. Use a different subclone or a different region of the gene as a labeled probe.

### High Background with FISH on Tissue Sections

#### Question

We have encountered high unspecific background (extra- and intracellular) when using fluorochrome-labeled probes in *in situ* hybridization.

#### Answer

This unspecific background may be caused by autofluorescence of tissue structures. Examples: Various tissues contain high amounts of extracellular matrix (collagen structures and elastic fibers in the heart or vessel samples) or may contain lipid droplets (lipofusion bodies in myocardial samples) with high autofluorescence. The problem with the autofluorescence of the matrix structure cannot be overcome. However, this feature may be used for better orientation in the tissue. To remove lipid droplets, delipidize the section in chloroform.

Avoid drying out of the sections. This always causes high background.

Background may also depend on the kind of fixation procedure used or overfixation.

Another hint from the following reference: Tautz (2002) Whole mount *in situ* hybridization for the detection of mRNA in *Drosophila* embryos. **Nonradioactive *In Situ* Hybridization Manual, p. 208 – 215, Third Edition.**

- ▶ The Anti-DIG antibody conjugate should be freshly preabsorbed for 1 hour against fixed material to prevent unspecific binding. The final working dilution of the antibody conjugate is 1 : 2000 in PBT. The preabsorption step should be adjusted accordingly.

**Example:** If ten reactions are processed in parallel, use about 200 µl material in 1 ml PBT with an antibody conjugate dilution of 1 : 200. This solution is then further diluted 1: 10 in the next step. Note: The diluted antibody solution can be reused for two or more stainings for a few days after dilution.

## 2. Fluorescence *In Situ* Hybridization (FISH)

### Unspecific Binding to Nucleolar Organizing Regions or Puffs

#### Question

Nearly all chromosomal bands are strongly and brilliantly fluorescing, in particular the nucleolar organizing region and large puffs are labeled. What could be the reason for this observation?

#### Answer

The reason is unspecific binding to chromosomal proteins caused by drying of the chromosome preparations during hybridization / washing procedures. So called “pseudohybridization” typically appears in local patches in the preparations. These regions may have dried out during handling, and as a consequence the DNA-probe and/or the antibody has been irreversibly linked to the chromosomes.

Another reason for obtaining more than the expected chromosomal loci can be that an inappropriate hybridization temperature or ionic strength in the hybridization solution has been applied. If the stringency of hybridization is significantly too low (greater than 25°C below the melting temperature) the hybridization reaction becomes less specific. More mismatches are tolerated and stretches of oligonucleotides are sufficient to bind the DNA probe.

The problem can be solved by increasing the stringency by higher hybridization temperature or decreasing the salt concentration during the washing steps.

### Scattered Irregular Background Spots on Chromosome Preparations

#### Question

There are many irregular spots of fluorescence scattered all over the chromosome preparation obscuring the “real” hybridization signal.

#### Answer

This is a frequently observed phenomenon when antibodies are stored over a long period of time. The reason is not immediately apparent. It seems that little clumps of precipitated antibodies are scattered around. It may help to centrifuge the antibody solution and take only the resulting supernatant in order to get rid of this “fluorescent dust”.

## 2. Fluorescence *In Situ* Hybridization (FISH)

### Strong Diffuse Fluorescence Signals Next to the Chromosomes

#### Question

There are strong diffuse fluorescence signals next to the chromosomes, sometimes forming a gradient towards the chromosome and the chromosomes seem to show no signal.

#### Answer

This phenomenon appears when the denaturation of the DNA of the chromosomes has gone too far. The result is an extraction of the DNA from the chromosomes (“spreading out”) similar to the picture shown by the following references.

Paulson, J. R. and Laemmli, U. K. (1977). The structure of histone-depleted metaphase chromosomes, *Cell* 12, 817 – 828.

Laemmli UK, Cheng SM, Adolph KW, Paulson JR, Brown JA, Baumbach WR. (1978) Metaphase chromosome structure: the role of nonhistone proteins. *Cold Spring Harb Symp Quant Biol.* 42, 351 – 60.

### Storage of Chromosome Preparations for FISH

#### Question

Can FISH preparations be stored over a longer period of time?

#### Answer

Yes, when the preparations are mounted in Glycerol with antifading reagents, these slides can be stored in the dark and at  $-20^{\circ}\text{C}$  for several weeks.

### Poor Chromosome Morphology after Hybridization

#### Question

The chromosome morphology is very poor after *in situ* hybridization, what can be the reason?

#### Answer

In particular the denaturation step is a harsh treatment for the chromosomes. The chromosomes have to be stabilized to survive this step with a reasonable maintenance of the chromosome morphology. One way to stabilize the chromosomes is a heat treatment in 2 x SSC for 30 minutes at  $70^{\circ}\text{C}$ . Make sure, that this temperature is really reached in the buffer within the cuvette containing the chromosomes.

The problem of poor maintenance of chromosome morphology is especially observed, when freshly prepared chromosomes are used. Storage of the preparation in ethanol or isopropanol for a longer period of time stabilizes the chromosomes and reduces the risk of destroying the chromosome morphology by the denaturation step.

## 2. Fluorescence *In Situ* Hybridization (FISH)

### Loss of Chromosomes after FISH

#### Question

After all steps of the FISH procedure there are no or only few chromosomes left on the slide.

#### Answer

Sometimes, depending on the glass slide used, the chromosomes do not stick properly to the glass surface. Some researchers use “coated” slides, for example poly-L-lysine coated slides are known to be more adhesive than uncoated slide. A much more trivial explanation is that the “wrong slide side with the chromosomes” has been wiped off during the procedure, which is, however, a typical mistake of beginners. A more serious explanation for the loss of chromosomes is the sticking of the chromosomes to the coverslip, which is flicked off in frozen state of the preparation. When the preparation is not really frozen during this step, then the probability is rather high that chromosomes stick to the coverslip. One possible solution is the treatment of the coverslips with silane and thus providing a hydrophobic surface.

### Fading Fluorescence Signals

#### Question

A hybridization signal is visible but the signal fades away quickly when inspected by the fluorescence microscope.

#### Answer

Most fluorescent dyes are bleached when the excitation light is on. The effect can be reduced very much by using antifading reagents (*e.g.*, 0.1% paraphenylene diamine in 50% glycerol) in the mounting solution. Bleaching is also a general problem in FISH. In all steps where the fluorescent dye is exposed to light, bleaching occurs. Therefore, only minimal light exposure of the dyes is recommended.

## 3. Probes / Probe Choice / Labeling

### General Considerations for Probe Choice

#### Question

Which probe type should I use for the different ISH applications?

#### Answer

For general recommendation concerning probe choice please refer to the **Nonradioactive *In Situ* Hybridization Manual, p.15, Chapter 1: Choosing the Right Labeling Method for your Hybridization Experiment** and the **DIG Product Selection Guide**.

### Probe Choice: PCR / RNA / Oligo Probes

#### Question

I recently prepared a 210 base pairs long DIG probe using the **PCR DIG Probe Synthesis Kit** for *in situ* hybridization. The technique worked only when the synthesized probe is used with a 1:3 proportion of labeled nucleotide, at 1:20 dilution in hybridization buffer, incubation at 42°C o/n. However, the reaction was faint. Alternatively, I prepared an internal oligoprobe (50 nucleotides contained in the original 210) with a 5' end DIG labeling. In this case, the ISH did not work at all. Can you suggest the best method for DIG labeling for improving the technique?

#### Answer

PCR labeled DNA Probes *e.g.*, via **PCR DIG Probe Synthesis Kit** should basically work for the detection of abundant transcripts or chromosome detection but if RNA probes are applicable, *e.g.*, for the detection of rare transcripts we always recommend the use of RNA probes (*e.g.*, **DIG RNA Labeling Kit** (SP6/T7)).

- ▶ The probe should be at least 300 base pairs long, the optimal length is 600 – 1000 base pairs.
- ▶ If you have to stick to the use of oligos – at least use tailed oligos: minimal 48mers. And a further hint: as described in our **Nonradioactive *In Situ* Hybridization Manual, p. 157, To increase the sensitivity of in situ hybridization procedures, use a mixture of oligonucleotide probes that are complementary to different regions of the target sequence.**

If you are interested in detailed protocols, please refer to our **Nonradioactive *In Situ* Hybridization Manual** or **DIG Application Manual for Filter Hybridization**.

### 3. Probes / Probe Choice / Labeling

#### Large DNA Probes are not Suited for ISH

##### Question

Can I use a 10 kb DIG-labeled DNA probe for *in situ* hybridization?

##### Answer

This depends on the type of ISH you perform – chromosomal detection or mRNA detection in tissue? For tissues, cells and detection of mRNAs (refer to FAQ “**Optimal Length of RNA Probes**”) we recommend the use of smaller DIG-labeled probes because these hybridize more efficiently to the target (better penetration of the probe into the tissue). Using our **DIG-Nick Translation Mix** the labeled fragments obtained in the standard labeling reaction show a length distribution maximum in the range of 200 to 500 nucleotides.

Alternatively the 10 kb DIG fragment could be sonified or digested with a restriction enzyme. On the other hand please note that a 10 kb large fragment possibly contains homologies to different target sequences – and therefore may not be specific. This could lead to unwanted unspecific or background signals. If the 10 kb fragment is derived from genomic sequence it could contain repetitive sequence elements, which could also disturb the specific outcome of the hybridization. In this case the probe should be verified via **Northern blot analysis**. For details on Northern blotting with the DIG system please refer to our **DIG Application Manual for Filter Hybridization, Chapter 3.2: Hybridization of RNA Probes to a Northern Blot**.

#### Alkali-labile DIG-labeled Nucleic Acid Probes

##### Question

I am working with PCR DIG-labeled DNA probes and want to use the probes for *in situ* experiments. The probe is 1 kb long, can DNA probes be hydrolyzed? Will the alkaline hydrolysis procedure written in the **Nonradioactive In Situ Hybridization Manual, p. 56**, for RNA probes cleave the DIG-NTP linkage, or does this require a higher pH? If the DIG link is cleaved by this procedure, do you know of any other hydrolysis method that could be used with alkali-labile DIG-dUTP?

##### Answer

While RNA is alkali-labile – it is not possible to hydrolyze DNA probes. In fact, the alkali-labile ester bond is destroyed from a pH of 11.5 upwards:  
4 mM NaOH / pH 10.5; 40 mM NaOH / pH 12-13; 400 mM NaOH / pH 14.

Since **PCR probes** can be generated in any size, it is easier to directly generate a probe of a appropriate size than to try to reduce the size artificially. However, the best way to reduce the DNA fragment size, if necessary, is an enzymatic digest. From our experience the best results are obtained in ISH using probes of 400 to 1000 base pairs.

In your case we recommend to try using the 1 kb PCR DIG fragment for your *in situ* as it is. If a higher sensitivity is required (rare mRNA detection) – single stranded anti-sense RNA probes are probes of choice. These are produced by **in vitro transcription** from a DNA template. A protocol can be found in the **Nonradioactive In Situ Hybridization Manual**.

## 3. Probes / Probe Choice / Labeling

### Probes for Rare mRNA Detection

#### Question

I want to detect a very rare mRNA in tissue sections. What kind of ISH-system would you recommend; what is the most sensitive ISH-system in your opinion?

#### Answer

We would recommend using **DIG-labeled cRNA probes** combined with the **Anti-DIG-AP/NBT/BCIP detection system**. This is the most sensitive nonradioactive ISH-system with a sensitivity comparable to that of radioactive ISH thus shorter exposure times (rather hours instead of days or weeks). If you are interested in detailed protocols please refer to our **Nonradioactive *In Situ* Hybridization Manual**.

### Vectors for *in vitro* Transcription

#### Question

Which is the best vector for *in vitro* transcription?

#### Answer

Many commercially available vectors are suitable for *in vitro* transcription. Important features are: T3, T7 or SP6 promoters on either side of the polylinker. The multiple cloning site should contain a good selection of unique restriction sites, especially those that leave 5'-overhangs. Avoid cloning your gene fragment into restriction sites that leave 3'-overhangs: RNA polymerases may wrap around and produce transcript of the opposite DNA strand, resulting in background in *in situ* hybridization experiments. Also, the vector should be high copy and should not be too large, so that the ratio of gene/vector is high. Commonly used vectors are pSPT18/19, pBSIIS+ or SK+ (Bluescript<sup>®</sup>, Stratagene), pGEM or pSPORT.

Roche Applied Science offers the **DIG RNA Labeling Kit** (SP6/T7) which already includes cloning/transcription vectors pSPT18 or pSPT19; Respective subclones are transcribed into RNA probes by T7 or SP6 RNA polymerase (also included in the kit).

### RNA Probe Preparation

#### Question

The digestion of my template is often incomplete. Do I need to worry about circular DNA interfering with the *in vitro* transcription?

#### Answer

Yes, circular or nicked plasmid DNA in the transcription reaction may interfere with transcription, making it less efficient and producing nonspecific transcripts. It is best to use only clean plasmid DNA\* to ensure that restriction enzyme digestion is complete. \*For example using the **High Pure Plasmid Isolation Kit**. Use 10 units of restriction enzyme per microgram DNA and digest for at least 3 hours. It is very important that the template is of high purity and free of contaminants such as salts, proteins, RNase, etc. Therefore it is highly recommended to gel-purify the linearized template and use a spin column for further clean-up (e.g., **High Pure PCR Product Purification Kit**). Phenol/chloroform extraction and ethanol precipitation is also recommended alterna-

### 3. Probes / Probe Choice / Labeling

tively. Resuspend the template DNA in RNase-free water (e.g., [Water, PCR grade](#)), check an aliquot on a gel for size, purity and concentration and use 1 µg of template in the *in vitro* transcription reaction. We recommend the use of e.g., [DIG RNA Labeling Kit](#).

#### Determination of DIG RNA Probe Labeling Efficiency

##### Question

How can I check for yield and incorporation of label after *in vitro* transcription?

##### Answer

You can use a dot blot comparing a standard of known concentration with your probe. DIG-labeled standards are supplied in the according kits e.g., [DIG RNA Labeling Kit \(SP6/T7\)](#). Make a dilution series with the standard and your probe (1:5 through 1:500 is a reasonable range) and spot 1 µl side by side on a [Nylon Membrane, positively charged](#). After UV-cross-linking develop the dot blot using blocking, antibody incubation and washing steps in analogy to the *in situ* hybridization protocol. The intensity of coloration will give you an approximation of probe yield. A detailed protocol is given in the Roche Applied Science [Nonradioactive In Situ Hybridization Manual, p. 202](#).

#### Optimal Length of RNA Probes

##### Question

When working with [single stranded RNA probes](#), what is the optimal length to get a most effective ISH detection?

##### Answer

In general the length of the probe should not be shorter than 250 bases (also dependent on the sequence and thus on the number of DIG-labeled nucleotides) and not longer than 1200 bases to guarantee most effective probe infiltration into the tissue. For larger probes please perform [alkaline hydrolysis procedure](#) as described in the [Nonradioactive In Situ Hybridization Manual, p. 55f, Chapter 4: RNA labeling by \*in vitro\* transcription of DNA with DIG, Biotin or Fluorescein RNA Labeling Mix](#).

## 3. Probes / Probe Choice / Labeling

### Alkaline Hydrolysis of RNA Probes

#### Question

What is **carbonate hydrolysis** for and what kind of probes require it?

#### Answer

Carbonate hydrolysis is a procedure for reducing the size of RNA probes to a desired length. The reason for this is that short RNA molecules penetrate the tissue and reach the target sequences more easily than long sequences. A suitable length for easy tissue access is 150 base pairs. Therefore, perform carbonate hydrolysis for all RNA probes that are longer than 200 – 300 base pairs. A protocol for carbonate hydrolysis and the formula for calculating the incubation time can be found in the **Nonradioactive *In Situ* Hybridization Manual, p. 55**.

### DIG RNA Labeling Performance – Important Hints

#### Question

What are the reasons for reduced labeling performance of *in vitro* DIG RNA labeling e.g., with the **DIG RNA Labeling Kit (SP6/T7)**?

#### Answer

If you used the **DIG RNA Labeling Kit (SP6/T7)** check functionality of the components using the linearized control template provided with the kit.

**Please note, that in most cases a decreased labeling efficiency can be improved following the recommendations mentioned below:**

- ▶ Repurify the linearized template DNA with the **High Pure PCR Product Purification Kit** or perform Phenol /Chloroform-extraction to exclude RNase contamination.
- ▶ A restriction enzyme creating a 3'overhang instead of a 5'overhang was used for linearization of the template plasmid. DNA templates with 3'-overhanging or blunt ends (in between case) may also yield unwanted transcripts of the “wrong” (opposite) DNA strand, caused by “run on” transcription. To avoid this, always use restriction endonucleases which create 5'-overhanging ends.
- ▶ Check by gel electrophoresis, if the transcript has the expected size:  
There are some DNA sequences that may cause the RNA polymerases to produce abortive or shortened transcripts. These problems may be overcome by recloning the fragment into a vector with the opposite direction of the polylinker and transcribing the same DNA-strand with a different RNA polymerase or transcribing the other strand of the template DNA.
- ▶ The amount of EDTA present in the labeling reaction is too high, use **water** or low TE buffer to resuspend the linearized template DNA.
- ▶ Check functionality of the kit components using the linearized control template that is provided with the kit (vial 3 or 4).

Further troubleshooting hints can be found in the **package insert** of the **DIG RNA Labeling Kit (SP6/T7)**.

### 3. Probes / Probe Choice / Labeling

#### Antisense Probes versus Control Sense Probes, Northern Blot

##### Question

Although the single stranded cRNA probes we are using are of appropriate length (about 800 bases) and efficiently labeled, we had problems with obtaining specific signals in our ISH procedure.

##### Answer

If you are sure that the signal cannot be specific and you have included all the necessary controls:

- ▶ Hybridization excluding a labeled probe to control background from the detection system
- ▶ Sense probe for controlling nonspecific binding of the probe

The probe itself may cause a “specific” problem. We would recommend performing a **Northern blot** analysis. The cause may arise due to your probe detecting an additional mRNA which is additionally expressed in your tissue sample. Please refer to the **example** attached or to the link at <http://www.roche-applied-science.com/ots/pdf/ish.pdf>.

#### Oligo Probe Labeling / Cacodylate Buffer

##### Question

I would like to label a probe to perform *in situ* hybridization and according to your protocol potassium cacodylate buffer is used. Where can I find a source for this reagent?

##### Answer

Roche Applied Science offers oligonucleotide labeling kits containing cacodylate buffer: **DIG Oligonucleotide Tailing Kit, 2<sup>nd</sup> generation** or **DIG Oligonucleotide 3'-End Labeling Kit, 2<sup>nd</sup> generation**. Alternatively we offer as a single reagent **Terminal Transferase, recombinant** supplied with 5x reaction buffer and separate CoCl<sub>2</sub> solution.

#### Recommendations for Oligos

##### Question

I am testing the **DIG Oligonucleotide Tailing Kit** to prepare a probe for mRNA localization by *in situ* hybridization. Any hints or ideas?

##### Answer

Especially for the detection of rare transcripts we always recommend the use of RNA probes (e.g., **DIG RNA Labeling Kit**).

If you have to stick to the use of tailed oligos: switch to a size of at least 48mers. And a further hint: as described in our **Nonradioactive *In Situ* Hybridization Manual, p. 157**, **To increase the sensitivity of *in situ* hybridization procedures, use a mixture of oligonucleotide probes that are complementary to different regions of the target RNA.**

If you are interested in detailed protocols please check out our **Nonradioactive *In Situ* Hybridization Manual** or the **DIG Application Manual for Filter Hybridization**.

## 3. Probes / Probe Choice / Labeling

### Oligos / Experimental Conditions

#### Question

Currently I am using your **DIG 3' End Labeling Kit** to label several oligos (26mers) and using your **DIG Nucleic Acid Detection Kit** to detect the ISH signals.

The situation for now is that the sense oligos (six oligos for six different mRNA targets) labeled similar cells types in the olfactory tissue as the antisense oligos did and the hybridization 'looked' specific. In a separate study, we used a nonsense oligo as a negative control but the resultant reaction came out the same as the sense control assay. In these attempts, stringency optimisation had appeared not to differentiate the antisense assay and sense control successfully. We use 2% SDS and do not include tRNA in the hybridization solution.

#### Answer

##### *I. Sensitivity / General recommendations*

- ▶ Is it absolutely essential to use 26mers? For the detection of rare transcripts, always use **riboprobes**. Furthermore, if the GC content of the 26mers is lower than 50%, this could be problematic. If it is necessary to use oligos would it be possible to switch to at least 48mers? And a further hint: as described in our **Nonradioactive *In Situ* Hybridization Manual, p. 157, To increase the sensitivity of *in situ* hybridization procedures, use a mixture of oligonucleotide probes that are complementary to different regions of the target RNA.**

Another useful reference is:

Rüger, B., Hasan, Q.; Dunbar, R.; Neale, T.J. (1995) **Detection of TNF- $\alpha$  mRNA in glomerular visceral epithelial cells by *in situ* hybridization with DIG-labeled synthetic oligonucleotides in cytokine research studies.** Biochemica No. 3, 27 – 29.

##### *II. Experimental procedures*

The signals obtained might be generally unspecific. Very important: Was a control performed without adding any probe? If possible always include a strong positive control in the experiments!

- ▶ Are these oligos really labeled efficiently with DIG? Was a spot test performed? The estimation of the labeling efficiency should be done according the detailed description given in the package insert of the **DIG Oligonucleotide 3'-End Labeling Kit, 2<sup>nd</sup> generation**.
- ▶ Recheck formamide concentration and hybridization temperature according to the formula on page 34 of the **Nonradioactive *In Situ* Hybridization Manual**.
- ▶ Including tRNA: It is very important to include some kind of carrier DNA to compete for nonspecific binding of the probe. This can be e.g., **tRNA** or **fish sperm DNA**.
- ▶ Recheck SDS concentration: 2% SDS in the hybridization buffer could lead to loss of tissue sections from the slides!

## 3. Probes / Probe Choice / Labeling

### Oligo ISH: Acetylation Step

#### Question

Is the acetylation step essential?

#### Answer

Acetylation using acetic anhydride in triethanolamine reduces the nonspecific binding of the probe to tissue and slide due to electrostatic forces – and is therefore essential for many tissue types. The opinion that for oligo ISH this step may be omitted is quite divergent.

### Influence of DIG-labeled Probe on OD / Probe Purification

#### Question

I am using the **DIG RNA Labeling Kit (SP6/T7)** from Roche Applied Science to generate riboprobes for *in situ* hybridization. I have tried to quantify the probe using OD<sub>260</sub>, but I do not get clear results. For instance, I obtain a low rate of 260 / 280 nm. Does DIG interfere with the measurement?

#### Answer

There is no influence of incorporated DIG on the OD<sub>260</sub> of nucleic acids, since only 1 in 30 nucleotides is DIG-labeled; ε-DIG is not extremely high at 260 nm, so the incorporation is negligible in OD<sub>260</sub>-measurement of RNA/DNA.

- ▶ Determination of labeling efficiency of the DIG labeling reaction via spot test is highly recommended.

Use a dot blot comparing a standard of known concentration with your probe. The standard is supplied in the kit. Make a dilution series with the standard and your probe (recommended concentration range is described in the **package insert** of the **DIG RNA Labeling Kit**) and spot 1 µl side by side on a membrane. After UV-cross-linking develop the dot blot using blocking, antibody incubation and washing steps in analogy to the *in situ* hybridization protocol. The detailed protocol is described in the **package insert**. The intensity of coloration will give you an approximation of probe concentration.

- ▶ Do not phenol/chloroform extract your DIG-labeled probe because it will partition into the organic phase. It is not essential to purify the reaction after DIG labeling and prior to diluting the probe in hybridization buffer for most probes and applications.

### 3. Probes / Probe Choice / Labeling

#### Recommendations for Special Templates (AT-RICH / AT-LOW)

##### Question

I have heard that varying the concentration of Digoxigenin-11-UTP in the transcription reaction may improve labeling efficiency for some probes. I would like to test this for my probe, but in the 10x-NTP-mix within the **DIG RNA Labeling Mix** the concentration of DIG-UTP is predetermined. What is the best procedure to vary the concentration of DIG-UTP?

##### Answer

Indeed, depending on the AT-contents of your probe it makes sense to reduce or increase the concentration of Digoxigenin-11-UTP in the *in vitro* transcription reaction. The reason is that, if the AT-contents of the template is unusually high, steric hindrance may occur, resulting in shorter transcripts and reduced hybridization with target sequences. Reducing the concentration of DIG-UTP can help in this case. If the AT-contents of the template is unusually low, increasing the concentration of DIG-UTP may ensure labeling of most nucleotides.

To vary the concentration of DIG-UTP, buy or prepare 100 mM stock solutions of each **ATP**, **GTP**, **CTP** and **UTP** and 10 mM **Digoxigenin-11-UTP** and mix according to the desired concentration.

Alternatively we offer **Set of ATP, CTP, GTP, UTP** Ribonucleoside Triphosphate Set.

Find a detailed overview on available nucleotides at: [http://www.roche-applied-science.com/sis/amplification/overview\\_products/overview\\_nucleotides.htm](http://www.roche-applied-science.com/sis/amplification/overview_products/overview_nucleotides.htm).

#### Probe Concentration: Recommendations for ISH

##### Question

What is the recommended probe concentration for *in situ* hybridization applications?

##### Answer

Since the DIG System is very sensitive, it is important to work with defined amounts of probe and template to ensure optimal results. Therefore it is a prerequisite to check the efficiency of each labeling reaction by determining the amount of DIG-labeled product via a spot test on a piece of **nylon membrane**.

For filter hybridization applications defined probe concentrations can be recommended (Table 1 from **Reference** Kruchen and Rueger). Due to the diversity of *in situ* applications probe concentration is described very differently in the literature.

The optimal probe concentration depends strongly on the individual probe (GC-content, length, labeling efficiency, abundancy (of expression) of the target).

- 1) The most important issue – determine the labeling efficiency via spot test as described in the respective package inserts of the DIG labeling kits (e.g., **DIG RNA Labeling Kit (SP6/T7)**).
- 2) **0.1 – 0.5 ng /  $\mu$ l / kb DIG-labeled RNA**. For a new probe try up to 5x higher or lower to find the best concentration.

### 3. Probes / Probe Choice / Labeling

#### “Double” Labeling With Different Haptens for Dual Detection

##### Question

I would like to perform double labeling for ISH detection of different probes – which methods do you recommend?

##### Answer

Usually double labeling requires 2 different detection methods: *e.g.*, **AP** detection and **POD** detection. **NBT/BCIP**, **INT/BCIP** and **Fast Red** are AP substrates. **DAB** detection can be used in combination with **POD**.

In most cases, the detection procedure using NBT/BCIP gives the strongest signals and should therefore be applied to the detection requiring the highest sensitivity.

##### *Some Tips*

For color detection the **DIG / AP / NBT/BCIP** system are perfectly combined using the **Biotin / POD / DAB** system.

For another example please compare to our **Nonradioactive *In Situ* Hybridization Manual, p. 164f, Detection of mRNA on paraffin-embedded material of the central nervous system with DIG-labeled RNA probes.**

A protocol describing double ISH / IHC with **Fast Red** can be found in: **Biochemica No. 3 / 1994 pages 24 – 26** or under the following link:

[http://www.roche-applied-science.com/ots/pdf/biochemica\\_394\\_p24\\_26.pdf](http://www.roche-applied-science.com/ots/pdf/biochemica_394_p24_26.pdf)

Some information **on double labeling with NBT/BCIP** or Fast Red in combination with a fluorescent substrate can be viewed under:

<http://www.biology.ucsd.edu/~davek/intro.html>.

Another recommendation: Combine the DIG/AP/BCIP system together with the Vector ABC detection system. This protocol works very well for the simultaneous detection of GM-CSF (ISH) and characterization of expressing cell types (ICH).

Reference: Plenz *et al.*, Granulocyte macrophage colony stimulating factor (GM-CSF) and type VIII collagen are codistributed during atherogenesis and GM-CSF transiently stimulated the expression of type VIII collagen mRNA by smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* 1999, 19:1658 – 1668.

If fluorescence labeling is to be combined with the DIG / AP / ECF System (Molecular Probes) for ISH and IHC, the fluorescence labeled secondary antibody of choice is described in the following reference:

Reference: Abouhamed *et al.*, Tropomyosin 4 expression is enhanced in dedifferentiating smooth muscle cells and during atherogenesis. **Eur. J. Cell. Biol.** 2003, 82:473 – 782.

## 4. Fixatives / Embedding / Pretreatment

### Basics on Tissue Fixation

#### Question

What impact does the fixation type have on the outcome of *in situ* experiments?

#### Answer

Maintaining tissue morphology and preventing loss of mRNA from cells are prerequisites of successful ISH experiments. Tissues can be either fixed via perfusion or submersion in the fixative. Perfusion usually has the advantage that tissue morphology and RNA integrity is maintained due to the fact that the fixative is more rapidly spread through the tissue / cells.

In addition, perfusion results in ISH data with low background due to clearance of blood cells from the tissue. Fixation by submersion should only be used when perfusion is not possible – for example with clinical samples or embryonic tissues.

### Differences Between Fixatives

#### Question

In the literature I find that different fixatives are used in different protocols for *in situ* hybridization. What are the differences between formaldehyde, glutaraldehyde, methanol, ethanol and mixtures with acetic acid in the use as fixatives?

#### Answer

There are two major groups of fixatives: 1) Cross-linking fixatives (*e.g.*, paraformaldehyde, formalin, glutaraldehyde) and 2) protein-precipitating fixatives such as ethanol or methanol mixed with acetic acid. The type of fixative used will affect ISH sensitivity. Cross-linking fixatives result in good morphology and good retention of target sequences, however, tissue permeability may be reduced. Precipitating fixatives give very good tissue permeability, but the quality of tissue morphology is reduced and target nucleic acids may be lost. For *in situ* hybridization 4% paraformaldehyde in PBS usually gives very good results with most tissue types. For certain tissue types (*e.g.*, plant leaves) that are very recalcitrant to penetration by fixative FAA is a good alternative (50% ethanol, 10% formalin, up to 5% acetic acid).

### Tissue Preparation / Tissue Fixation for Blood Smears

#### Question

What kind of fixation method do I need to use for *in situ* hybridization on blood smears?

#### Answer

A commonly used fixation and dehydration procedure for blood smears and cytopins is 4% formaldehyde in PBS for 10 minutes followed by dehydration in 70%, 95% and 100% ethanol 1 minute each and air dried.

## 4. Fixatives / Embedding / Pretreatment

### Basics on Embedding / Sectioning

#### Question

Do you recommend cryostat sections of frozen tissue or paraffin-embedded tissue sections?

#### Answer

Either cryostat sections of frozen tissue and paraffin-embedded tissue sections can be used for ISH. Excellent results can be obtained with cryostat sectioning, so that different fixatives for immunocytochemistry and hybridization can be used in adjacent sections. In general, sectioning of embedded tissue has several advantages including better preservation of tissue morphology, the ability to cut thin sections and the ease with which the sample can be orientated and serial sections obtained. Paraffin wax is the most popular embedding medium since sections of down to 1  $\mu\text{m}$  can be cut and fully dissolved and washed out from the tissue prior to hybridization. 6  $\mu\text{m}$  sections are commonly used.

However, paraffin embedding requires more tissue processing and some researchers have observed that for certain applications this results in RNA loss and lower ISH signal.

Reference: *In Situ Hybridization. A practical Approach* Edited by DG Wilkinson IRL Press, Oxford University Press, 1994.

### Methacrylate Embedding / Quality Important

#### Question

We tried ISH with single stranded cRNA probes on methacrylate embedded sections without success. The probes worked well on cryosections. What would you suggest to get the ISH going on methacrylate sections?

#### Answer

Very important is the quality of the methacrylate embedded tissue. As with other embedding techniques there are several steps (handling of the tissue prior to fixation, fixation, dehydration and embedding steps) in which the mRNA may be degraded or lost. If the quality of the tissue and sections is beyond question, the most crucial steps are – not to lose the sections (e.g., use precoated slides) and- to provide for optimal penetration of the probe into the tissue. This is achieved by carefully adjusting the digest with **Proteinase K**.

## 4. Fixatives / Embedding / Pretreatment

### Sectioning Problems

#### Question

I often encounter problems when sectioning paraffin-embedded tissue. For example, the sections break up or disintegrate, split, scratch or shred. Also, sometimes they roll up tightly rather than forming a ribbon. What can I do to improve the sections?

#### Answer

When paraffin sections break up or disintegrate, the tissue sample may be too large for sectioning or the tissue is not embedded well (the fixative did not penetrate all the way resulting in the tissue becoming dry and brittle in the centre during dehydration, and the wax does not fill the cells), or sections have floated on the water for too long. Solutions are to 1) cut smaller tissue samples, 2) try different fixation regimes (e.g., use different fixative or vacuum infiltration), 3) reduce the length of time sections float on water.

When sections split, scratch or shred along the ribbon, one reason could be that the blade is chipped (even the smallest chips may cause problems) or dirty. Another reason may be that the tissue contains hard components such as cell walls or bone, or the material is not embedded well, resulting in dry areas that scratch and break the ribbon. To alleviate these problems examine the blade under binoculars for damage, clean the blade regularly and change when necessary.

When paraffin sections roll up tightly, the reason could be that the blade is not adjusted to the right angle on the microtome (it should be 4°) or it needs cleaning with 95% ethanol and distilled water.

### Eosin Y Dye for Orientation During Sectioning

#### Question

When I embed my tissue samples (plant material of 1 mm diameter) in paraffin, it is very hard to locate the tissue in the solid wax block because it is virtually transparent. It is also very difficult to orientate the block on the microtome for sectioning in the desired plane. What can I do to avoid this problem?

#### Answer

During the dehydration series after fixing the tissue, add 0.05% EosinY to the 100% ethanol steps. EosinY is a strong nonspecific dye, soluble in ethanol and water. The tissue attains a bright red color and will be easy to locate in the wax block. The tissue sections are stained a pale red color, which will disappear completely during rehydration and not interfere with the *in situ* hybridization.

## 4. Fixatives / Embedding / Pretreatment

### Loss of Tissue Sections from Slides / Subbing of Slides

#### Question

The problem I encounter very often is that the sections fall off the poly-L-lysine coated slides. What is the reason for this and how can I avoid this problem?

#### Answer

Sometimes, depending on the glass slide used, the tissue does not stick properly to the glass surface. An additional explanation for the loss of tissue is the sticking of the tissue to the coverslip.

#### ► Coverslips:

We recommend the treatment of the coverslips with silane and thus providing a hydrophobic surface.

#### ► Pre-treated Slides:

Many researchers use “coated” slides, for example poly-L-lysine coated slides. A very good alternative to poly-L-lysine coated slides are pre-treated slides such as Superfrost Plus (Manzel) or ProbeOn Plus (Fisher) microscope slides. To improve attachment of paraffin sections to the slides (all types), we recommend heating the slides to 42°C for several hours and additionally at 25 to 30°C overnight.

### Losing Sections / Proteinase K Digestion

#### Question

We have lost some of our sections after including a digest with proteinase K. Moreover, remaining sections displayed unsatisfactory morphology. There is quite a wide range of **Proteinase K** concentrations described in the literature. Do you have recommendations?

#### Answer

There are several potential reasons for loss of sections:

- the time of digest is too long
- the concentration of proteinase K is too high.

If **Proteinase K** is necessary for efficient probe infiltration its concentration must be carefully adjusted. Most important for the optimal digest is whether or not the **Proteinase K** solution has been predigested. In our experience predigested proteinase K solution is 10-times more reactive than proteinase K, which is not predigested. These differently treated kinds of proteinase K solutions may also explain to some extent the tremendous variability in concentrations in the literature. Moreover, to provide for steady activity of proteinase K solution, the solutions should be either freshly prepared or stored in small aliquots at –15°C to –25°C (avoid repeated cycles of freezing and thawing).

Moreover, the optimal concentration strongly depends on:

- the kind of tissue used
- the thickness of the sections
- if the tissue was embedded (and what kind of embedding procedure was used)

► We recommend our ready to use **Proteinase K** solution – simply dilute to your desired concentration.

## 4. Fixatives / Embedding / Pretreatment

### Proteinase K Pretreatment

#### Question

After *in situ* hybridization the tissue often looks damaged: however, before the experiment and after rehydration tissue integrity seems fine. The color of the signal is also rather a pale, diffuse blue that cannot easily be assigned to individual cells. What could be the reason for this?

#### Answer

During the *in situ* hybridization procedure the tissue undergoes a series of treatments to make it permeable to RNA probe. One of the critical treatments is the proteinase K step. For each tissue type the concentration of proteinase K and the duration of incubation have to be adjusted to achieve optimal results. Try a series of concentrations ranging from 0.1 µg/ml to 0.5 µg/ml for cryosections and 1 to 20 µg/ml for resin or paraffin sections using RNase-free **Proteinase K**.

### Proteinase K Overdigestion May Result in Nonspecific Background

#### Question

Although we were successful in obtaining a specific ISH-signal after introducing an additional **Proteinase K** digest into our ISH-protocol, the quality of the sections was markedly reduced. Moreover, we got some nonspecific background.

#### Answer

Overdigestion may result in the occurrence of nonspecific background. Reduce the concentration of proteinase K and/or the time of digest. Include an additional fixation step after the proteinase K digest.

- ▶ We recommend our ready to use **Proteinase K** solution – simply dilute to your desired concentration.

### Proteinase K May Result in Weak Signals

#### Question

ISH was used to detect a mRNA strongly expressed in our tissue. We received a specific signal but the intensity was very low. Therefore, we tried to improve the signal intensity in our ISH by enhancing probe penetration into the tissue using a proteinase K digest without success. We even lost the signal entirely (although the tissue structure was still satisfactory).

#### Answer

Sometimes **Proteinase K** will cause washing out of the mRNA during the later steps of the prehybridization procedure. We strongly recommend adding an additional fixation step directly after the digest. This step also provides for better preservation of morphology in the section.

- ▶ We recommend our ready to use **Proteinase K** solution – simply dilute to your desired concentration.

## 4. Fixatives / Embedding / Pretreatment

### Acetylation Reduces Nonspecific Background

#### Question

In my *in situ* hybridization results I have a lot of nonspecific signals. I have heard that an acetylation step can help to reduce the background. How does this work and would you recommend it?

#### Answer

Acetylation using acetic anhydride in triethanolamine reduces the nonspecific binding of the probe to tissue and slide due to electrostatic forces – and is therefore highly recommended for many tissue types.

Most protocols include acetylation as one of the tissue pretreatment steps.

Acetylation neutralizes positively charged molecules such as basic proteins, and prevents nonspecific binding of the probe to the slide when poly-L-lysine-coated slides are used. Acetylation also removes endogenous biotin, which can otherwise cause background signal if a biotinylated probe is used.

### DEPC Addition Not Necessary for Xylene or Ethanol

#### Question

Should DEPC be added to xylene or ethanol for dewaxing?

#### Answer

No, xylene and ethanol are RNase-free and do not need to be treated with DEPC.

## 5. Hybridization and Blocking

### DEPC Treated Water is Recommended for all ISH Buffers

#### Question

Many protocols recommend to use only DEPC-treated water in the *in situ* hybridization procedure. What is DEPC and where can I buy it?

#### Answer

DEPC is diethylcarbonate and you can order it from chemical suppliers such as Sigma (Cat. Nr. D 5758). It is used to make water RNase-free. RNases are extremely stable enzymes and may survive even autoclavation.

In the fumehood, add 1ml of DEPC to 1 liter of deionized, distilled water while stirring. Stir for at least 2 hours. Autoclave DEPC-water for 20 – 30 minutes to evaporate residual DEPC. After cooling to room temperature the water is ready to use. For RNA *in situ* hybridization, we recommend to use DEPC treated water to prepare all aqueous solutions.

Note: For protection of mRNA in *in vitro* transcription reactions we recommend using **Protector RNase Inhibitor**.

### Hybridization Buffer Recommendations

#### Question

There is a broad range of different hybridization buffers described in the literature. Some show a very simple composition, whereas others are of a more complicated composition. What is the reason for these conspicuous differences? Which buffer do you recommend when using DIG-labeled single stranded RNA probes?

#### Answer

The variability of the hybridization buffers depends on the kind of DIG probe used, but as general advice – besides using an appropriate buffer system – we would strongly recommend using blocking agents such as **tRNA**, **Fish Sperm DNA** or **BSA** to avoid non-specific binding of the DIG-labeled probe to the tissue samples – 50% **Formamide** to sufficiently reduce the temperature to the ISH and to preserve the optimal tissue structure.

When using single stranded DIG-labeled RNA probes the following buffer works perfectly: 50% formamide, 2x SSPE buffer, 1 mg/ml fish sperm DNA, 0.5 mg/ml yeast tRNA, 1 mg/ml BSA.

- Especially for *in situ* hybridization you should use freshly deionized formamide. Please note that due to slowly recommencing re-ionisation formamide has to be deionized if any yellow color occurs. This is also true if the product was deionized by the supplier before purchase. Deionization of formamide is described in standard literature, for example in Sambrook *et al.*, Molecular Cloning, page 1.102 or in Current Protocols.

## 5. Hybridization and Blocking

### Formamide Recommendations

#### Question

What type of formamide is recommended for use in ISH?

#### Answer

Especially for *in situ* hybridization you should use freshly deionized **Formamide**. Please note that due to slowly recommencing re-ionisation formamide has to be deionized if any yellow color is observed. This is also true if the product is deionized by the supplier before purchase. Deionization of **Formamide** is described in standard literature, for example in Sambrook *et al.*, Molecular Cloning, page 1.102 or in Current Protocols.

### Hybridization Buffer for ISH Not Commercially Available

#### Question

Does Roche Applied Science provide a hybridization buffer as they do for filter hybridization?

#### Answer

Since conditions of ISH vary so differently between the different kinds of ISH-systems, the composition of the buffers has to be carefully adapted to the ISH system (kind of tissue, fixation procedure, embedding procedure, thickness of the section or tissue, kind and length of the probes) and a general ISH buffer system cannot be defined.

### DIG Easy Hyb Not Recommended for ISH

#### Question

Can **DIG Easy Hyb** also be used for *in situ* hybridization?

#### Answer

**DIG Easy Hyb** can be applied to all types of nucleic acid blot hybridizations, but is not suitable for *in situ* hybridization, since some of the buffer components may affect the tissue. For detailed protocols concerning hybridization buffers please refer to our **Nonradioactive *In Situ* Hybridization Manual**.

### General Blocking Considerations

#### Question

What general recommendations are available concerning blocking reagents for ISH?

#### Answer

There are a large variety of blocking solutions commercially available (*e.g.*, **Blocking Reagent**). Some researchers also include low fat milk powder. For general recommendation concerning blocking methods please also refer to the **Nonradioactive *In Situ* Hybridization Manual, p. 25, Chapter 2: Details of the Technique**.

## 5. Hybridization and Blocking

### Blocking Reagent for Whole Mounts

#### Question

Can I use **Blocking Reagent** for whole mount *in situ* hybridization with DIG-RNA probes?

#### Answer

- ▶ During the *in situ* hybridization procedure unspecific background is more efficiently reduced by adding **tRNA** from Roche Applied Science or **Fish Sperm DNA** to the hybridization solution.
- ▶ The **Blocking Reagent** is used to decrease the background in nonradioactive hybridization and detection of nucleic acid hybrids. 1) Blocking prior to the detection procedure is optional – you can either use the blocking reagent or serum from the animal (*e.g.*, sheep) the antibody used is from. Please also compare to our **Non-radioactive *In Situ* Hybridization Manual, p. 25** and **p. 212**. The antibody may be preadsorbed for 1 hour with the whole mount species to reduce the background.
- ▶ For immunological detection in ISH please refer to the protocol in the **Non-radioactive *In Situ* Hybridization Manual, p. 164**.

Protocol for “**Use in Immunological Detection** in ISH”.

(*e.g.*, DIG-RNA probes & tissue sections; please also compare to **Nonradioactive *In Situ* Hybridization Manual, p. 168**).

After last washes continue to immunological detection:

Put slides in a tray suitable for 8 slides with 30 ml of buffer.

Incubate as follows (and change trays rather than just solutions and wash the extra set of trays between incubations to avoid background):

- a) Buffer 1 [100 mM Tris-HCl pH 7.5, 20°C; 150 mM NaCl] 5 minutes;
- b) Buffer 2 [buffer 1 with 0.5% (w/v) **Blocking Reagent**] 1 h;
- c) Buffer 3 [buffer 1 with 1% (w/v) **BSA**; 0.3% (v/v) Triton X-100] 1 hour;
- d) Drain off excess of buffer 3 (do not let the tissue become dry) and pour 100 µl buffer 4 [buffer 3 with **Anti-DIG-AP** 1:1000 to 1:3000 depending on your target) on the tissues (or in 15 ml in glass tray). Prepare freshly before use!
- e) Incubate 1 hour at RT in a moist chamber;
- f) Wash 4 times in buffer 3 (20 minutes);
- g) Equilibrate in buffer 1 for 5 minutes;
- h) Incubate 5 minutes in buffer 5 (100 mM Tris-HCl (pH 9.5, 20°C; 100 mM NaCl; 50 mM MgCl<sub>2</sub>);
- i) Perform color reaction (*e.g.*, NBT/BCIP), leave in the dark, cover trays to avoid evaporation; check in appropriate time intervals; stop enzyme reaction and wash off background, return slides back into the stainless steel rack and wash.

## 5. Hybridization and Blocking

### tRNA Recommendations for ISH

#### Question

Which tRNA do you recommend for *in situ* experiments?

#### Answer

Adding tRNA as a competitor to pre- and hybridization solution prevents nonspecific binding of the probe. Different protocols employ a variety of different competitors *e.g.*, **Fish Sperm DNA** of nonspecific binding of the probe. There is no special tRNA recommended – for references please refer to the **Nonradioactive *In Situ* Hybridization Manual** and order tRNA of your choice. For example **tRNA from *E. coli* MRE 600** (RNase negative).

### DTT Purpose During Hybridization

#### Question

Is the addition of **DTT** necessary? What's the major purpose of the addition during *in situ* hybridization

#### Answer

DTT is commonly used as a protective agent, stabilizer, inhibitor, stimulator of enzyme activity, anti-toxicant and reducing agent.

#### *DTT in Hybridization Solution*

In general, it is essential to include **DTT** in the probe / hybridization solution only if you are working with 35S probes, because they can crosslink via disulfide bonds and lead to formation of aggregates which cause ugly background signals. For nonradioactive ISH DTT may be omitted. However, if it is present, ensure that it does not interfere with the experiment.

### Use Clean Coverslips to Prevent Tissue Damage

#### Question

For *in situ* hybridization, I add probe on top of the tissue and cover it with a coverslip to avoid evaporation during the overnight incubation at 55°C. However, when the coverslips are removed in the first stringency wash, I find streaks of damage through the tissue. Also, sometimes I observe a patchy signal or no signal at all, even though the tissue was covered evenly with probe. How can I avoid these problems?

#### Answer

It is very important that the coverslips are absolutely clean. Even the smallest specks of dust can damage the tissue in the process of sliding off after overnight hybridization. Some suppliers' coverslips are reliably clean (*e.g.*, EMS), others are not. Coverslips can be cleaned in an acetone bath, but make sure that all residual specks are removed and do not precipitate elsewhere on the coverslips.

The patchy or absent signal may result if coverslips are not entirely RNase-free. To remove all RNase, wrap little stacks of coverslips in several layers of aluminium foil and bake overnight in a dry oven at 180°C. Cool down and store until used. If the tin foil is not damaged, the coverslips will be ready to use for many months.

## 6. Detection / Counterstaining / Mounting

### ISH Detection Using NBT/BCIP

#### Question

I am interested in the substrate **NBT/BCIP** for alkaline phosphatase for *in situ* hybridization. Is it possible to detect the color precipitate in the fluorescent microscope at a certain wavelength like it is the case for **Fast Red**?

#### Answer

Usually the slides stained with **NBT/BCIP** are viewed under a light microscope. Depending on the counterstain used you may also visualize the signals using a microscope that is equipped with a UV source (“using Epifluorescence to reveal the underlying tissue”). Very faint signals can be more easily detected under dark field.

Please note that NBT/ BCIP is **not** a fluorescent precipitate like **Fast Red** which can absorb and emit light at special wavelengths.

If you are interested in developing a fluorescence signal – combine DIG / AP with ECF substrate system instead of NBT/BCIP. For more information concerning this topic, please refer to FAQ “**Double labeling With Different Haptens for Dual Detection**” under **Section 3. Probes / Probe Choice / Labeling**.

### Duration of Color Detection

#### Question

Do you recommend leaving the color reaction overnight? What are the reasons for false positives after 8 to 10 hours of color reaction?

#### Answer

It is convenient to leave the color reaction overnight. But this duration time also is strongly dependent on probe and tissue. You will have to empirically determine if this incubation overnight is compatible with your specific probe & tissue. Good washing is of course also important when doing so. When performing an overnight color reaction always keep your coplin jars air tight and in the dark! **NBT/BCIP** is very sensitive to air – unspecific precipitates will form. Always check for trapped air bubbles between the slides in your jar and carefully remove them.

## 6. Detection / Counterstaining / Mounting

### MgCl<sub>2</sub> in Detection Buffer

#### Question

I am performing ISH using probes labeled with digoxigenin to detect RNAs. After the hybridization I use Anti-DIG-antibodies conjugated to alkaline phosphatase (AP) and the immunological detection is performed with **NBT/BCIP**. I have two questions: 1) Why does the buffer for the color reaction contain MgCl<sub>2</sub> at 50 mM? 2) What is the objective of this concentration?

#### Answer

The reason why MgCl<sub>2</sub> is included into the detection buffer is that originally it was postulated that alkaline phosphatase has a central ion (Mg<sup>2+</sup>). But since this is zinc – MgCl<sub>2</sub> is no longer included in the detection buffer for immunological detection of DIG-labeled probes for filter applications. BUT for *in situ* hybridizations all the protocols still include MgCl<sub>2</sub> and no one has really tested to see what the results would be without MgCl<sub>2</sub> in the buffer. If you happen to make a side by side comparison of your ISH with and without MgCl<sub>2</sub> we would of course be very interested in your results.

### Duration of Color Detection / Bleeding of Signal into Surrounding Tissue

#### Question

When developing the ISH color reaction overnight, we observed some bleeding of the color precipitates into the surrounding tissue. Do you have a suggestion for avoiding this?

#### Answer

This problem occurs mostly when frozen tissue samples are used. This tissue is fragile and not as well preserved as paraffin- or plastic-embedded sections. However, this general background does not interfere with the sensitivity or specificity of the DIG-probe used.

Another cause may be that the mRNA of interest is very strongly expressed. In this case the small amount of the color precipitate can cause some diffusion of the signal into the surrounding tissue. To avoid this bleeding the time of development has to be carefully adjusted to your needs (if not inconvenient, a development overnight should be avoided).

### Expired NBT/BCIP

#### Question

What effects have reagents such as the chromogen **NBT/BCIP** been reported to have on ISH using oligos when used after their expiration date? The NBT/BCIP I am using is about one month over the expiration date.

#### Answer

If the NBT/BCIP has expired it might not be sensitive enough for ISH detection. Please prepare the **NBT** and the **BCIP** freshly for highest sensitivity, and adjust the pH of the detection solution carefully to pH 9.5.

## 6. Detection / Counterstaining / Mounting

### Weak and Brown instead of Blue Signals

#### Question

Using **NBT/BCIP** for alkaline phosphatase, instead of a final blue color precipitate I obtain a faint brown-purple reaction product in the tissue after RNA *in situ* hybridization. The reaction is specific for the given mRNA, but the color is different than expected and really weak.

#### Answer

##### *I: Brown-Purple instead of Blue Signals*

With *in situ* hybridization the color of **NBT/BCIP** precipitate can vary from blue to brown or purple. The final color depends primarily on the abundance of target mRNA in the tissue, but is also influenced by probe length and labeling intensity. The pH of the detection solution (reaction buffer for alkaline phosphatase) can also play a role and should be carefully adjusted to pH 9.5.

In general, the more abundant the target RNA, the stronger the corresponding signal, resulting in a deeper blue color precipitate.

If a deeper blue or purple signal is desired, try our **BM Purple Substrate**.

##### *II: Weak Signals*

If the probe is properly tested (gel and spot assay were OK), works well in **Northern Hybridization** and only gives weak signals in ISH (provided the protocol is working), we would start looking at the quality of the tissue sections or the detection procedure:

- ▶ Testing different probe concentrations:  
Evaluate higher amounts of probe in the hybridization mix: e.g., 1, 2, 4, 8 µl of the 50 µl diluted standard reaction per 50 µl hybridization solution.
- ▶ Include a strong positive control.
- ▶ Test an alternative protocol using chloroform instead of **Proteinase K**, glycine.

We know that experienced *in situ* researchers do not like to change protocols. But it may be worth to test a completely different protocol for this approach.

**Please note:** Important for DIG *in situ* is that there is no drying out of the slides after the pretreatment steps and prior to the hybridization! Drying of the slides causes unspecific background.

## 6. Detection / Counterstaining / Mounting

### Dark Blue NBT/BCIP Precipitates

#### Question

I am using free floating brain sections in the detection buffer (500 microliters) and I am adding the appropriate amount of NBT/BCIP. Typically the incubation period is 18 hours (overnight) at 25°C. This time the detection buffer with **NBT/BCIP** was very dark blue with a lot of precipitate! It was the first time that this occurred. Although I have signal in my sections I was wondering about this peculiar event?

#### Answer

- ▶ Please check the pH of the detection buffer, it must be in the range of pH 9.5, 20°C; minimize the exposition of the detection solution to the air (use air tight coplin jars).
- ▶ Can you observe precipitates already in the concentrated **NBT/BCIP** stock solution? If precipitates occur in the color substrate solution, they can be removed by warming up the solution to 50°C.
- ▶ If the precipitates do not dissolve, spin the tube prior to use because this may cause background; spinning will not reduce the overall sensitivity.

### High General Blue Background / NBT/BCIP Detection

#### Question

I have used DIG-labeled probes for some time with very good success. But we had to change to a new tissue system (paraffin-embedded) and ever since I have a very high general blue background after using the normal alkaline phosphatase (NBT/BCIP) detection system. What causes this background?

#### Answer

The cause for high general nonspecific background in this system may be overfixation of the tissue. This usually results in a general blue staining of the whole tissue. Nevertheless, if you cannot avoid this – because the tissue you use is from a source you cannot change – this background should not interfere with the specific signal.

### Counterstains

#### Question

Which counterstains are recommended for nonradioactive *in situ* hybridization in combination with **Anti-DIG-AP** detection / **NBT/BCIP** ?

#### Answer

It is a well known phenomenon by histologists that **NBT/BCIP** is not compatible with the classical counterstains. NBT/BCIP signals should not be mounted with xylene containing mounting media (*e.g.*, DPX) because these could lead to crystal formations of the color precipitates. Unfortunately classical counterstains like Eosin need xylene containing mounting media.

## 6. Detection / Counterstaining / Mounting

The following mounting reagents are specifically on the market for mounting sections with **NBT/BCIP** signals: Crystalmount from Biomedica or Vectamount or Immunomount from Vector Laboratories. The same companies also offer organic counterstains which are compatible with these mounting media (e.g., Vector Methyl Green, Vector Nuclear Fast Red).

The results of combining a particular counterstain with any of the mounting media primarily depends on the type of tissue used for **NBT/BCIP** color detection. Staining adjacent slides with or, without NBT/BCIP detection with a typical counterstain and to mount the slides with the classical Xylene containing mounting medium. This allows the direct comparison of stained tissue with or, without signal.

*Customer Recommended Protocol\* for the Preparation of a mounting medium:*

Glycerol gelatine:

- 100 ml of 0.2 M phosphate buffer pH 7;
- Na azide 200 mg;
- gelatine 15 g, stir until dissolved;
- glycerol 100 ml.

Keep at 37°C, add a drop to the slide and coverslip. After hardening of the mounting media the signal is said to last for several years without fading of the NBT/BCIP precipitate.

*\*Note: “Customer Recommended Protocols” were developed in customer labs and were not validated by Roche Applied Science.*

*Roche Applied Science is not liable for the contents and the successful execution of the protocols. The display of customer protocols on the Roche Applied Science homepage is only a service of Roche Applied Science to facilitate exchange of experience within the research community.*

### Fading of NBT/BCIP Signals

#### Question

Are there any specific recommendations to prevent fading of **DIG NBT/BCIP** signals for *in situ* hybridization experiments?

#### Answer

It is pretty unusual that fading of **NBT/BCIP** is observed in *in situs*. Usually fading is an issue for fluorescence ISH.

Its important to consider that slides with **NBT/BCIP** signals should never be embedded in xylene- based mounting media because these could lead to crystal formations of the color precipitates.

► Please compare to FAQ **“Counterstains” for Customer recommended mounting protocol.**

## 7. Troubleshooting

### General

#### Question

What are your general **recommendations** for troubleshooting *in situ* results – if a poor signal is observed or if the background is too high?

#### Answer

##### A. If a poor signal is observed, then:

- 1) Check whether the probe is correctly labeled (see FAQs Section 3. **Probes / Probe Choice, / Labeling**).
- 2) Check **Proteinase K**\* digestion. Depending on the tissue and the Proteinase activity, it may be necessary to perform rather extensive Proteinase K digestions. Set up a series of digestion conditions and test these.
- 3) Check for RNase contamination of the solutions. Treat the solutions with diethylpyrocarbonate before use, in particular the PBT solution before addition of **Tween 20**.
- 4) Drosophila Embryo Specific Hint: Check under the binocular microscope whether the vitellin membranes are fully removed after the methanol step.

\* Roche Applied Science recommends ready to use **Proteinase K**, solution

##### B. If the background is too high, then:

- 1) Prolong the washing step, because of insufficient washing each step may be done for longer times.
- 2) Test higher dilutions of the antibody.
- 3) Perform longer preabsorption of the anti-digoxigenin complex. (Please also refer to FAQs **Background Problems on Bone / Tooth Tissue / Levamisole**.)
- 4) Include levamisole in the staining solution. Levamisole acts as a potent inhibitor for endogenous lysosomal phosphatases.

Note: These are, however, usually not a problem in early Drosophila embryos.

- 5) Increase detergent concentration in the PBT. **Tween 20** may also be replaced by SDS.
- 6) Include a xylene treatment step after the fixation.

### High Background Reasons / Quick Checklist

#### Question

I observe high background in my ISH results – what should I consider to check?

#### Answer

High Background in ISH can have multiple reasons – in short:

- ▶ Probe labeling efficiency/ Probe concentration / Probe sequence itself
- ▶ Sticky Tissue / Pretreatment *e.g.*, Proteinase K conc. not optimal
- ▶ Permeabilization issue

- ▶ Overfixation
- ▶ Antibody conc. / Antibody reactivity: sometimes preabsorption with special tissue necessary
- ▶ Hybridization / Washing stringency
- ▶ Color reaction – overdevelopment
- ▶ Important for DIG *in situ* is that there is no drying out of the slides after the pre-treatment steps and prior to the hybridization! Drying of the slides causes unspecific background to arise.
- ▶ Endogenous AP activity

For more further informations please refer to FAQs under **Troubleshooting**.

### Signals Washed Away / Whole Mounts

#### Question

Our customer has successfully used the combination of **Digoxigenin + Anti-DIG-AP + NBT/BCIP** for labeling whole mount ISH of mouse embryos. The labeling and signal are absolutely OK.

After paraffin-embedding of labeled embryos and using sections in the ISH – the signal is substantially decreased. For paraffin-embedding we used methanol + isopropanol and 1,2,3,4-tetrahydronaphtalen. The sections were deparaffinized using a standard protocol including xylene, graded series of ethanol and water.

Do you have any recommendation how to stop the loss of the strong specific signal when using paraffin-embedded tissue?

#### Answer

It is recommended to perform postfixation of the whole mount embryos after **NBT/BCIP** detection. Suggestion: use 3% paraformaldehyd in PBS buffer supplemented with 1% glutaraldehyd. This should prevent signal decrease in further steps *e.g.*, paraffinization; sectioning etc. Without fixation – **the steps where most color signal is washed away are the ethanol wash series and especially the 70% ethanol washing step**. The use of xylene should not be the problem. For mounting sections organic mounting media are recommended, *e.g.*, Merckoglass, Paramount, Euparal.

### Endogenous AP Activity / Levamisole Treatment

#### Question

How can endogenous AP activity be inhibited to prevent unspecific background in tissue sections?

#### Answer

High background during ISH (caused by endogenous AP) can be reduced by using Levamisole, because intestinal calf AP is inhibited only little by Levamisole whereas other mammalian APs are inhibited effectively. Please refer to this [Link](#) or the protocol in the **Nonradioactive *In Situ* Hybridization Manual, p. 156**.

## 7. Troubleshooting

### Background Problems on Bone / Tooth Tissue / Levamisole

#### Question

We encounter background problems when using **Anti-DIG-AP, Fab fragments** following an established protocol for *in situ* hybridization on hard tissue (bone / tooth).

#### Answer

The following steps should be checked:

- ▶ How was tissue embedding performed – did you have problems with other detection methods? Bones have a very high content of endogenous alkaline phosphatase. Please always inhibit the endogenous AP via Levamisole treatment. Please refer to this [Link](#) or the protocol in the **Nonradioactive In Situ Hybridization Manual, p. 156**.
- ▶ Always check if the probe reacts as expected in **Northern hybridization**. If Northern blots already show background problems – *in situ* analysis is also prone to problems. In this case choose a different probe spanning a different or similar region / modify the size.
- ▶ Did any of the components you used change during the whole ISH procedure: *e.g.*, tissue pretreatment (*e.g.*, **Proteinase K**, hybridization conditions [probes, probe concentrations], washings including RNase treatments). The other steps which should be checked are antibody concentration (*e.g.*, too high), washing duration (*e.g.*, too short), length of the color reaction (*e.g.*, too long).

Recommendation: Perform a negative control – incubating the substrate alone with the section should not cause any background. A further negative control which we recommend is to perform the whole ISH procedure with the sense probe or no probe in parallel to the antisense probe and then go through the full detection process including antibody binding. The results might tell if perhaps the antisense probe used is sticky (hybridizing unspecifically).

### High Granular Background with Riboprobes

#### Question

I am using riboprobes in *in situ* and I am experiencing a high (granular) background. The experiment was working well a few months ago but now problems occur. I have tried to reduce the probe concentration without success. Then I preabsorb the **Anti-DIG-AP** antibody with mouse embryo powder and incubate in sheep serum + **BSA**. I also use Levamisole to reduce background AP activity. Detection is with **NBT/BCIP** prepared from separate products.

#### Answer

- ▶ Has a negative control (without probe, but all other steps identically treated) been performed?
- ▶ Is this granular background specific to the particular probe? Do you see it only with one specific probe? Have you included a positive control (a probe giving a tissue-specific expected pattern)?
- ▶ If you see this background for all controls there might be something going wrong during the pretreatment steps (maybe **Proteinase K** digestion was not optimal).

## 7. Troubleshooting

- ▶ Please try to make out the difference between the former experiments and the recent ones. There could be something / some new batch of a component? Or one of the products might be expired?
- ▶ In the **Nonradioactive *In Situ* Hybridization Manual** you can find detailed protocols for tissue sections and riboprobes. Maybe you can compare the conditions with those you used.
- ▶ Quality of the tissue or the sections: are the sections freshly cut for ISH or stored for some time before use? The quality of sections can decrease upon storage. This is especially crucial for cryosections.
- ▶ Endogenous AP activity  
High background during ISH (caused by endogenous AP) can be reduced by using Levamisole, because intestinal calf AP is inhibited only little by Levamisole whereas other mammalian APs are inhibited effectively. Please refer to this [Link](#) or the protocol in the **Nonradioactive *In Situ* Hybridization Manual, p. 156**.

### General

High Background in ISH may not only be due to endogenous AP activity but could also be due to the following reasons ( see also FAQ **High Background Reasons / Quick Checklist**):

- a) Probe concentration too high / sticky probe
  - b) Pretreatment steps not efficient (*e.g.*, **Proteinase K**)
  - c) Stringency of hybridization or washing steps too low
- ▶ Did you include some sort of **negative and positive control**? You should always run a negative control in the way that you perform all the steps the same but you only omit the probe. Likewise you should also include a sense control which should also show no specific signals. Additionally, if available use a positive control: a probe which is known to give a specific pattern in your tissue is great for proving that the conditions chosen are OK.

### Heart Samples: Unspecific Vesicular Background

#### Question

After using heart samples for ISH we observed some unspecific “vesicular” blue background after performing the alkaline phosphate (NBT/BCIP) detection procedure.

#### Answer

Heart and also some other tissues may accumulate lipid droplets intracellularly. When using the alkaline phosphate (NBT/BCIP) detection procedure on cryosections some of the color precipitate is trapped in these lipid droplets. This problem is solved by delipidizing the sections in chloroform (10 minutes at RT) prior to the prehybridization procedure.

## 7. Troubleshooting

### High Background Mammary Gland / Levamisole Treatment

#### Question

Using your product **Anti-DIG-AP, Fab fragments** for detection of *in situ* hybridization on sections I obtain high background on my sheep tissue sections (mammary gland). I do not know if Levamisole helps, because it does not inhibit intestinal calf AP-s and the sheep's endogenous AP must be very similar to the calf AP due to the high degree of homology between these animals. Where is the conjugated AP on Anti-DIG derived (from calf)? Could you give me an advice to decrease high background?

#### Answer

Indeed the AP is derived from calf intestine.

High background during ISH (caused by endogenous AP) can be reduced by using Levamisole, because intestinal calf AP is inhibited only little by Levamisole whereas other mammalian APs are inhibited effectively. You must also keep in mind the large excess of AP you have at the specific region the probe binds to. Please refer to this [Link](#) or the protocol in the **Nonradioactive *In Situ* Hybridization Manual, p. 156**.

High background in ISH may not only be due to endogenous AP activity but could also be due to the following reasons:

- ▶ Probe concentration too high / sticky probe
- ▶ Pretreatment steps not efficient (e.g., **Proteinase K**)
- ▶ Stringency of hybridization or washing steps too low

Further recommendations:

- ▶ Include a negative control: e.g., perform all the steps but omit the labeled probe.
- ▶ Include a sense control which should show no specific signals.
- ▶ If available use a positive control: a probe which is known to give a specific pattern in your tissue is great for proving that the conditions chosen are OK.

Please check out the protocols in the **Nonradioactive *In Situ* Hybridization Manual, p. 149f**, e.g., [the results for all type of gland tissues](#).

### Unspecific Background at Border of Sections

#### Question

After performing the alkaline phosphate (NBT/BCIP) detection procedure we observed some unspecific background at the border of our sections. Could you give some suggestions as to what may cause this kind of background?

#### Answer

- ▶ Please make sure that the sections do not dry out during the detection procedure. However, besides the detection procedure, which may cause this background (though this is rather unlikely, since detection is usually performed in glass vessels), other steps may account for the problem described, *e.g.*, the hybridization procedure.
- ▶ Make sure that the section is fully covered with prehybridization/hybridization buffer during hybridization. A reduction of the volume and drying out of the sections during these steps may be the underlying reason.  
We would recommend:
  - Assuring that the box used for hybridization is well sealed.
  - Performing the hybridization in a humid chamber containing a solution composed of the same buffer and formamide concentration as the hybridization buffer.
  - Covering the sections with coverslips, or more conveniently with parafilm.

## 8. References

### DIG Product Selection Guide

### Nonradioactive *In Situ* Hybridization Manual

### DIG Application Manual for Filter Hybridization

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## 8. References

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Further References

**DIG System *In Situ* Hybridization Reference List**

## 9. Selection of Useful Web-links

LabFAQS – A compendium of frequently asked questions in life science research.  
(Protocols, Buffers, Formulas, Tables for DNA, RNA, proteins and cells)

<http://www.roche-applied-science.com/labfaqs/>

FISH Guide and Troubleshooting

<http://info.med.yale.edu/genetics/ward/tavi/FISHguide.html>

FISH – (Fluorescence *In Situ* Hybridization)

<http://www.accessexcellence.org/RC/VL/GG/fish.html>

FISH – (References)

<http://www-biology.ucsd.edu/~davek/inforefs.html>

IHC World

[http://www.ihcworld.com/protocol\\_database.htm](http://www.ihcworld.com/protocol_database.htm)

Immunohistochemistry – *In situ* Hybridization

<http://home.no.net/immuno/>

*In situ* hybridization protocols

<http://www.genedetect.com/>

The Jackson Laboratory (Plant *in situ*)

<http://jacksonlab.cshl.edu/>

The Power and Versatility of *In Situ* Hybridization

<http://martin.parasitology.mcgill.ca/insituhybridization/insitu.htm>

Optimizing *In Situ* Hybridization Protocols

[http://www.ambion.com/techlib/tb/tb\\_507.html](http://www.ambion.com/techlib/tb/tb_507.html)

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## 11. Ordering Information

Please refer to our **DIG Product Selection Guide** available as a hard copy or as a pdf file. For a complete list of products please view our Catalog or Website at [www.roche-applied-science.com](http://www.roche-applied-science.com).

## 12. Feedback Corner

This compendium of FAQs is a living document. These questions are also available on our website under our technical online help at [www.roche-applied-science.com/support](http://www.roche-applied-science.com/support). If you have suggestions for improvements or encounter difficulties or if you want to share your experience with other researchers please contact our [www.roche-applied-science.com/support](http://www.roche-applied-science.com/support) or [Bettina.Kruchen@roche.com](mailto:Bettina.Kruchen@roche.com) directly.

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