

## 10 Tips for Optimizing *In Situ* Hybridization (ISH)

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Enzo Life Sciences provides more than 40 years of experience in the manufacturing and supply of research kits, biochemicals and biologicals. As Scientists Enabling Scientists, we are happy to provide simple but useful tips for improving daily tasks as well as the overall quality of your research. With this in mind, here is a comprehensive list of tips for achieving high quality data by in situ hybridization, a widely used biological technique providing the end user with accurate localization of endogenous, bacterial or viral nucleic acids such as DNA, mRNA, and microRNA in metaphase spreads, cells, and tissue preparations.



### 1. Suitable Probe

As hybridization can take place between complementary deoxyribonucleotides or ribonucleotides, either DNA- or RNA-based probes (riboprobe) can be used to localize DNA or RNA in a given sample. However, RNA-RNA hybrids are more stable than RNA-DNA hybrids, which in turn are more stable than DNA-DNA hybrids. Double-stranded DNA probes are easy to prepare, label, and work with in the laboratory, while single-stranded RNA probes are uniform

in size, achieve high incorporation of label, and form highly stable RNA-RNA hybrids. Also, single-stranded oligonucleotide probes can easily be chemically synthesized and labeled to high specific activity nowadays. Alternatives using peptide backbones or locked nucleic acids (LNA) are available today as well, which may enhance hybridization efficiency and stability.

## 2. Appropriate Label

Radioactive labeling of probes for tissue microarrays should be done with  $^{35}\text{S}$ -NTP to avoid bleeding of the signal. If the aim is to visualize the probes in combination with the surrounding cells or tissue, which is most often the case, you may, e.g., choose between a wide range of fluorescent dyes that allow for direct detection of the probes. Alternatively, you could also use intermediaries, such as biotin or digoxigenin, and a chromogen to produce staining that can endure the stress of time, much like in immunohistochemistry (IHC).

## 3. Correct Labeling Technique

Nick translation or random-primed labeling are the methods of choice for generating long, double-stranded DNA probes, while *in vitro* transcription from vectors containing RNA polymerase promoters is used for the production of riboprobes. Oligonucleotides can either be directly labeled during their synthesis or labeled nucleotides may be added to their ends using terminal deoxynucleotidyl transferase or T4 polynucleotide kinase.

## 4. Appropriate Detection Method

Direct detection is possible due to fluorescent labels that can be introduced during FISH probe synthesis and detected by fluorescence microscopy. This way, multiplexing can easily be envisaged, as two or more different probes labeled with different fluorophores can be visualized at any single time. Biotin labels can be detected with avidin from egg white or streptavidin from the bacteria *S. avidinii* while digoxigenin can be paired with anti-digoxigenin antibodies. Both indirect labeling methods can finally be visualized with either alkaline phosphatase (AP) or horseradish peroxidase (HRP), which can react with specific substrates to produce a chromogenic precipitate for CISH.

## 5. Optimize Proteinase K Digestion

Proteinase K digestion is a critical step for successful ISH as insufficient digestion will result in a diminished hybridization signal. On the other hand, if the sample is over digested, tissue morphology will be poor or completely destroyed, making localization of the hybridization signal impossible. Therefore, optimal assay concentrations of Proteinase K will vary depending

upon the tissue type, length of fixation, and size of the tissue. In general, a good starting point for ISH applications is the use of 1–5 µg/mL Proteinase K for 10 min at room temperature. Determination of the optimal Proteinase K digestion conditions should be done by a Proteinase K titration experiment followed by hybridization with the probe of choice. The Proteinase K concentration that produces the highest hybridization signal with the least disruption of tissue or cellular morphology is the one you should choose for your assays.

## 6. Enhance Hybridization Conditions

The aim is to obtain hybridization only between the probe and its target and thus to achieve the highest specificity possible. Hybridization specificity (stringency) is primarily driven by the degree of homology between the probe and target nucleic acid sequences, probe concentration, temperature and time of hybridization, and concentration of monovalent cations present in the hybridization solution. In particular, hybridization temperature, typically ranging between 55 and 62 °C, should be optimized carefully for achieving highly specific results. Formamide allows hybridization at temperatures significantly lower than the actual melting temperature of a probe-target-hybrid and thus may assist in the conservation of the morphology of samples. For the demonstration of genes, COT sequences are routinely included to reduce non-specific hybridization to repetitive DNA sequences.

## 7. Optimize Post-hybridization Conditions

The reactions are followed by post-hybridization washes of increasing stringency to dissociate imperfect matches, which leaves only specifically bound probe on target sequences. If you are struggling with high background in your ISH assays, you can further reduce background by digesting nonspecifically bound probes with nucleases. Use S1 nuclease for DNA probes and RNase for RNA probes before proceeding to the detection step.

## 8. Modify Washing Steps if Using DNA Probes

DNA probes can provide equivalent sensitivity to RNA probes, however, DNA probes do not bind as tightly to the target molecules in your samples. Therefore, formaldehyde should not be used in the post hybridization washes when using DNA probes.

## 9. Optimize Detection

When using biotinylated probes, be aware that biotin is also endogenously produced in cells and tissues. Endogenous biotin may thus lead to nonspecific staining as (strept-)avidin-based detection systems will bind to all biotin molecules that are freely accessible in your samples. In those cases, you either need to block endogenous biotin by adding excess (strept-)avidin to

your samples prior to probe hybridization or you should use digoxigenin instead of biotin as your probe label. Digoxigenin is a non-radioactive immune tag isolated from the Digitalis plant and as such, is unlikely to be detected by biological materials other than specific anti-digoxigenin antibodies. Hence, use of a digoxigenin label allows for probe detection with high affinity, sensitivity, and most importantly, specificity.

## **10. Change Reagents Frequently**

In order to get the most reproducible and valid data, triethanolamine and acetic anhydride should be replenished once every 2-3 weeks and 10% neutral buffered formalin (NBF) should be changed out every 3-4 days.