ISH Protocol

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Introduction

Following an in situ hybridization (ISH) protocol allows researchers to detect and localize specific nucleic acid sequences within fixed tissues or cells. ISH is a powerful molecular technique that is essential in both research and diagnostic settings, from gene expression studies to chromosomal mapping. By visualizing RNA or DNA using an in situ method, the ISH protocol reveals spatial patterns of gene expression that are often lost in homogenized assays.

Whether you're performing a fluorescence in situ hybridization (FISH) procedure or using chromogenic detection, success depends on high-quality reagents and optimized protocols for each step, from fixation to signal development.

Browse Our Buffers

Materials & Reagents

Here's everything you need to carry out our ISH protocol. In addition to Boston BioProducts' high-quality buffers and reagents, you'll need the following materials and equipment:

- Glass slides or coverslips
- Humidified hybridization chamber
- Forceps and slide racks
- Incubator or hybridization oven (37–45°C)
- Water bath (95°C for probe denaturation)
- Coplin jars or staining dishes for washes
- Fluorescence or brightfield microscope

Reagent	Product Name	CAT#
Wash Buffers	10X Phosphate Buffered Saline, PBS Phosphate Buffered Saline-Tween, PBST Tris Buffered Saline, TBS (20X) Tris Buffered Saline-Tween, TBST Distilled Water	BM-220 IBB-171R BM-300X IBB-181R WT-055
Blocking Buffers	Bovine Serum Albumin (BSA) Casein (3%, TBS) Casein (3%, PBS)	P-753 IBB-130 IBB-120
Hybridization Buffers	Saline Sodium Citrate (20X) Denhardt's Solution (50X) Denhardt's Solution (100X)	BM-240 BM-130 BM-130X
Detergents & Permeabilizers	Triton X-100 Tween-20 (100%) Proteinase K Solution (20mg/mL) Sodium Dodecyl Sulfate, SDS (20%) NP-40 Lysis Buffer (2X)	P-924 P-934 P-1460 BM-230 BP-119X

Reagent	Product Name	CAT#
Fixatives	Paraformaldehyde (4%, PBS) Paraformaldehyde (4%, Phosphate Buffer) Paraformaldehyde (3.7%, Fixative Solution) Paraformaldehyde (4%, PBS with Mg & EGTA) Formalin (10%, Phosphate Buffer) Formalin (10%, Acetic Acid Solution)	BM-155 BM-698 BM-158 BM-156 BM-705 BM-707
Probe & Antibody Preparation Buffers	Tris-HCL Buffer (1 M, pH 8.0) EDTA (0.5 M, pH 7.4) Sodium Chloride (1 M) Sodium Acetate (20 mM)	BBT-80 BM-711 BPS-405 BPS-346
Substrate Buffers	Custom Buffers	

Boston BioProducts' Protocol

The following ISH protocol provides a generalized workflow that can be adapted for both chromogenic and fluorescence-based detection methods. Always tailor parameters like probe concentration, hybridization temperature, and stringency washes to your specific tissue type and target nucleic acid.

Fixation and Permeabilization

Fixation preserves tissue structure and nucleic acid integrity, while permeabilization allows probe access. These steps are critical for achieving specific, localized hybridization signals and minimizing morphological artifacts.

⚠ **For paraffin sections**: Ensure sections are fully deparaffinized and rehydrated before fixation. Residual wax inhibits probe binding.

1. Fix samples in 4% paraformaldehyde (PFA) for 15–30 minutes at room temperature.
2. Wash in PBS to remove residual fixative.
3. Permeabilize tissue using 0.1% Triton X-100 or treat with Proteinase K for RNA targets.

💡 To reduce background, especially in chromogenic detection workflows, you can include an optional acetylation step after permeabilization. Acetylation chemically blocks positively charged amines in tissue, which helps prevent nonspecific probe and antibody binding.

Pre-Hybridization Blocking

Pre-hybridization conditions the sample and blocks nonspecific binding sites. This helps ensure that only your probe binds to its complementary target, improving signal clarity and reducing background.

1. Formulate your pre-hybridization buffer. Here's a recipe you can use for 100 mL:

Component	Stock Concentration	Final Concentration	Volume to Add
Formamide	100%	50% (v/v)	50mL
SSC	20X	1X	5mL
Heparin	50mg/mL	50µg/mL	100µL
Salmon sperm DNA	10mg/mL	100µg/mL	1mL
SDS	10% (w/v)	1% (w/v)	10mL
Tween-20	20% (v/v)	0.1% (v/v)	0.5mL
RNase-Free Water	-	-	To 100mL total

> ⚠ Filter-sterilize through a 0.2 µm filter and denature the salmon sperm DNA at 90–100°C for 10 minutes before adding.

> Warm your pre-hybridization buffer before applying it. Pre-warmed buffer helps maintain consistent temperature and tissue permeability.

2. Incubate samples in the pre-hybridization buffer at 37–45°C for 30–60 minutes.

> ⚠ Probe concentration can significantly impact signal quality and background. Start with the recommended dilution and optimize based on tissue type and detection method.

3. Cover with a coverslip and incubate overnight (16–18 hours) at 37–45°C in a humidified chamber.

Hybridization

This is the core of the ISH protocol—when the labeled probe binds to its complementary DNA or RNA sequence. Precise temperature control and adequate incubation time are essential for specificity and sensitivity.

1. Denature the labeled probe at 95°C for 5 minutes, then place on ice.
2. Apply the denatured probe diluted in hybridization buffer.

> ⚠ Probe concentration can significantly impact signal quality and background. Start with the recommended dilution and optimize based on tissue type and detection method.

3. Cover with a coverslip and incubate overnight (16–18 hours) at 37–45°C in a humidified chamber.

Stringency Washes

Washing removes unbound or weakly bound probes and reduces background signal. Adjusting stringency through temperature and salt concentration helps retain specific hybridization while eliminating noise.

1. Gently remove coverslips.
2. Wash in 2X SSC for 5–10 minutes at room temperature.
3. Follow with 0.1X SSC at 55–65°C for 15 minutes.

> ⚠ Use a hybridization oven or water bath to maintain wash temperatures — stringency is temperature-sensitive.

Signal Detection and Imaging

After hybridization and stringency washes, visualization of the probe-target complex is achieved through either chromogenic or fluorescent detection. In a chromogenic ISH protocol, the signal is enzyme-amplified and visible under brightfield microscopy. For fluorescent detection, such as the FISH protocol in situ, directly or indirectly labeled probes allow for sensitive imaging with fluorescence microscopy.

For Chromogenic ISH:

1. Block in Casein or BSA-based buffer.
2. Incubate with enzyme-conjugated antibody (e.g., anti-DIG-AP).
3. Develop with substrate (e.g., NBT/BCIP or DAB) until signal appears.
4. Rinse in PBS and counterstain with hematoxylin if desired.
5. Mount with an aqueous or permanent medium and allow to dry.
6. Image using brightfield microscopy.

> The indirect ELISA protocol provides signal amplification but may introduce cross-reactivity with the secondary antibody. It is useful for detecting antibodies in a sample, such as in serological testing.

For FISH:

1. Rinse in PBS or SSC.
2. Counterstain with DAPI.
3. Mount with antifade medium to preserve fluorescence.

4. Image using a fluorescence microscope with appropriate filter sets.
5. Adjust exposure settings to minimize background and avoid signal saturation.

> Always include a negative control (no probe) and positive control tissue to validate signal specificity.

> ⚠ Capture images immediately after mounting. Over time, fluorescence can diminish, and chromogenic precipitates may diffuse.

Troubleshooting & Optimization Tips for ELISA Protocols

Even with a well-designed in situ hybridization procedure, issues like background staining or weak signal can arise. Here's how to troubleshoot common problems and optimize your ISH workflow for reliable results.

My background signal is too high

The problem:
Non-specific probe binding, insufficient washes, or inadequate blocking can all contribute to elevated background in your ISH protocol.

What to do:

- Increase stringency of your post-hybridization washes (e.g., higher temperature, lower SSC concentration)
- Include blocking agents like salmon sperm DNA or tRNA in your hybridization and pre-hybridization buffer
- Consider adding an acetylation step after permeabilization to block positively charged amines

My signals are weak

The problem:
Low probe binding efficiency may result from poor tissue accessibility, probe degradation, or insufficient concentration.

What to do:

- Optimize probe concentration and follow the manufacturer's instructions for the in situ method used
- Ensure effective permeabilization (Proteinase K or detergent-based)
- Check RNA or DNA integrity before hybridization—degraded samples yield a low signal

My staining is uneven

The problem:
Patchy or inconsistent staining often results from poor probe distribution or drying during hybridization.

What to do:

- Apply the probe evenly and ensure full coverage of the sample
- Use coverslips and a properly sealed humidified chamber to prevent evaporation
- For fluorescent detection, such as the FISH protocol in situ, avoid air bubbles that can distort signal distribution

I see non-specific signals

The problem:

Off-target binding or tissue damage can cause a misleading signal in your in situ hybridization protocol.

What to do:

- Confirm probe specificity with proper negative and positive controls
- Include a no-probe control to identify background staining
- Use RNase or DNase digestion to validate whether the signal is RNA- or DNA-dependent

Why Boston BioProducts?

Boston BioProducts offers a comprehensive selection of high-quality reagents specifically formulated for ISH protocols. From fixation to final imaging, our buffers are designed for consistency, low background, and compatibility with both chromogenic and fluorescence-based detection systems.

Need a specialized formulation? We also provide custom buffer services to support unique tissue types, detection chemistries, or workflow needs. Whether you're developing a FISH protocol in situ or troubleshooting background in chromogenic ISH, our products—and our team—are here to help you optimize every step.

FAQs

What's the difference between hybridization and prehybridization buffers?

A prehybridization buffer is applied before the probe to block nonspecific binding sites and reduce background. It typically contains formamide, SSC, Denhardt's solution, and additives like salmon sperm DNA or heparin. In contrast, the hybridization buffer includes the labeled DNA or RNA probe and maintains optimal conditions for specific hybridization. Both are essential for a successful in situ hybridization protocol and must be carefully formulated.

Can I use the same ISH protocol for both chromogenic and fluorescence detection?

Yes. The core in situ hybridization procedure (fixation, permeabilization, hybridization, and washes) remains the same. The difference lies in detection: chromogenic ISH uses enzyme-linked antibodies and a colorimetric substrate (e.g., NBT/BCIP), while the FISH protocol in situ uses fluorescent labels or antibody amplification for signal detection. Both methods require careful buffer selection for optimal performance.

Do I need RNase-free reagents for RNA ISH protocols?

Absolutely. When performing an in situ method targeting RNA, even trace amounts of RNase can degrade your samples and compromise results. RNase-free reagents are essential during tissue preparation, hybridization, and wash steps to maintain RNA integrity. This is especially critical for detecting low-abundance transcripts using fluorescent or chromogenic readouts. We offer RNase-free formulations of core reagents like PBS, and can also prepare custom buffers in RNase-free formats to support sensitive RNA detection in your in situ method.