October 2017

miRCURY® LNA® miRNA Detection Probes Handbook

Detection of miRNA by FFPE *in situ* hybridization (ISH) using double-labeled, LNA-enhanced probes

Sample to Insight

QIAGEN

Contents

Kit Contents

Product	Cat. no.	Amount supplied
miRCURY LNA miRNA Detection Probe (1 nmol)	339111	1 nmol oligonucleotide, dried down in tube format
miRCURY LNA miRNA Detection Probe (10 nmol)	339112	10 nmol oligonucleotide, dried down in tube format
miRCURY LNA miRNA Custom Detection Probe (1 nmol)	339115	1 nmol oligonucleotide, dried down in tube format
miRCURY LNA miRNA Custom Detection Probe (10 nmol)	339116	10 nmol oligonucleotide, dried down in tube format
miRCURY LNA miRNA ISH Buffer and Control	339459	Scramble miRNA Negative Control Probe, U6 snRNA Positive Control Probe, 25 ml 2x Formamide-free miRNA ISH buffer, 1.25 ml Proteinase K Solution
miRCURY LNA miRNA ISH Buffer Set (FFPE)	339450	25 ml 2x Formamide-free miRNA ISH buffer, 1.25 ml Proteinase K Solution

Storage

The miRCURY LNA miRNA Detection Probes and the miRCURY LNA miRNA ISH Buffer Set (FFPE) are shipped at room temperature. Immediately upon receipt, the miRCURY LNA miRNA ISH Buffer should be stored at 2–8°C. Store the Proteinase K Solution at room temperature (15–25°C). For storage longer than one year or if ambient temperature often exceeds 25°C, we suggest storing Proteinase K Solution at 2–8°C. Store the DIG- and FAM-labeled miRCURY LNA miRNA Detection Probes at –15 to –30°C or below. Under these conditions the probes are stable for at least 6 months. It is recommended to store the probes in aliquots and to avoid multiple freeze-thaw cycles. Do not store in frost-free freezers with automatic thaw-freeze cycles.

Intended Use

The miRCURY LNA miRNA Detection Probes are intended for molecular biology applications. These products are not intended for the diagnosis, prevention or treatment of a disease.

All due care and attention should be exercised in the handling of the product. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of miRCURY LNA miRNA Detection Probes is tested against predetermined specifications to ensure consistent product quality.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view and print the SDS for each QIAGEN kit and kit component.

Introduction

miRCURY LNA miRNA Detection Probes, miRCURY LNA miRNA ISH Buffer and Controls, and the miRCURY LNA miRNA ISH Buffer Set (FFPE) are designed for specific miRNA detection by *in situ* hybridization (ISH). This handbook includes our "One-Day miRNA ISH Protocol," which was developed specifically for ISH on formalin-fixed paraffin embedded (FFPE) tissue samples. The protocol can also be easily adapted for ISH on fresh frozen tissue.

The One-Day miRNA ISH Protocol minimizes time-consuming optimization steps and enables a fast and optimal miRNA ISH analysis using a colorimetric, antibody-based development system for the DIG-labeled probes. The protocol also covers each step of the FFPE ISH procedure, including tissue sectioning, recommended selection of miRNA-specific, positive and negative control probes, incubation intervals and temperatures, miRCURY LNA miRNA Detection Probe concentrations and substrate incubation. We also include a list of recommended equipment and reagents required to establish and optimize miRNA ISH in the lab.

Overview of the One-Day miRNA ISH Protocol

The One-Day miRNA ISH Protocol is designed for detection of miRNA in FFPE tissue sections. The protocol takes advantage of the non-mammalian hapten digoxigenin (DIG) or carboxyfluorescein (FAM) and has been optimized to fit into a one-day experimental setup. During the protocol, the miRNAs are demasked using Proteinase K, which allows the access of double-DIG or double-FAM labeled LNA probes to hybridize to the miRNA sequence (Figure 1). The digoxigenin and FAM haptens can then be recognized by a specific anti-DIG antibody or anti-FAM antibody, respectively, that is directly conjugated with the enzyme alkaline phosphatase (AP). AP converts the soluble substrates 4-nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3′-indolylphosphate (BCIP) into a water- and alcohol-insoluble, dark-blue NBT-BCIP precipitate. Finally, the nuclear counterstain is applied to the sections to allow better histological resolution.

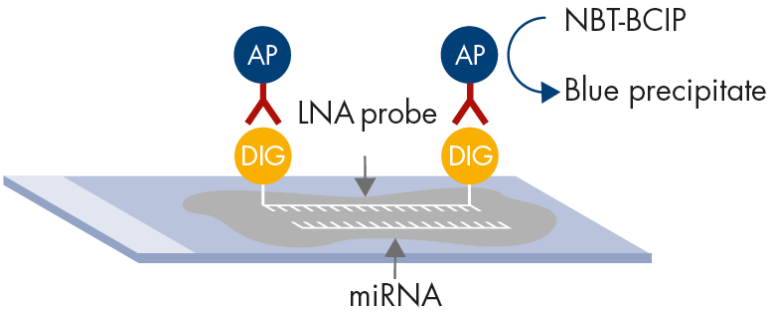
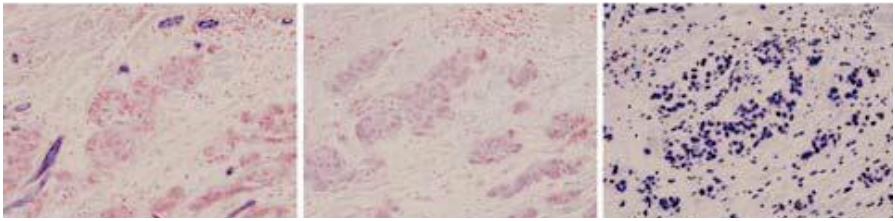


Figure 1. Principle of the miRCURY LNA miRNA ISH Optimization Kit (FFPE). First, the tissue is "opened" using Protease K. In the hybridization step, the DIG-labeled LNA probe binds specifically to its target miRNA. Alkaline phosphatase (AP)-conjugated anti-DIG antibodies are then added. This step is followed by NBT-BCIP development and optional counterstaining with Nuclear Red.

Optimization of the ISH procedure is divided into three steps:

1. Optimization of the protocol parameters with the miRCURY LNA Detection U6 snRNA control probe (single-DIG or single-FAM labeled) by adjustment of hybridization temperature and Proteinase K treatment. A robust signal obtained with a low concentration of the U6 snRNA probe (e.g., 0.1–1.0 nM) indicates that the sensitivity is in the range suitable for detection of endogenous miRNAs.
2. Control study using the optimized protocol parameters with the miRCURY LNA miRNA Detection positive control probe and miRCURY LNA miRNA Detection Scramble-miR negative control probe (both double-DIG or double-FAM labeled). A strong, specific ISH signal should be obtained with minimal background. Further optimization may be required for Proteinase K treatment and hybridization temperature to minimize background staining.
3. Detection of the miRNA of interest using the appropriate miRCURY LNA miRNA Detection Probe (double-DIG or double-FAM labeled). Figure 2 shows a typical result of the miRNA ISH procedure. In this example, a specific hsa-miR-126-3p ISH signal is seen in endothelial cells. As expected, no signal is observed with the Scramble-miR negative control probe. Finally, overall nuclear staining is seen with the LNA U6 snRNA positive control probe.

Figure 2. In situ hybridization on consecutive sections from FFPE tissue sample with human breast cancer. The miRCURY LNA miRNA Detection Control Probes and the miRCURY LNA miRNA ISH Buffer Set (FFPE) were used, along with the miRNA-specific miRCURY LNA miRNA Detection Probe hsa-miR-126-3p.



hsa-miR-126-3p, 40 nM | Scramble-miR, 40 nM | U6 snRNA, 0.1 nM

Once optimized, the protocol allows exceptionally clear and specific detection of miRNA at the cellular and partly subcellular level, due to high signal-to-noise ratio. An excellent histological resolution is obtained in a wide range of tissue samples. This makes the miRCURY LNA miRNA Detection system ideal for a variety of applications, ranging from study of miRNA localization and developmental miRNA regulation to the examination of functional studies for biomarker discovery in clinical specimens and other biopsy material. The robustness of the procedure makes it advantageous for both high-throughput ISH analysis, as well as individual miRNA localization studies.

The choice between double-DIG and double-FAM labeled probes

The One-Day miRNA ISH Protocol was originally developed for double-DIG labeled LNA probes (see Jorgensen et al, Methods 2010). However, the miRCURY LNA miRNA Detection Probes are available with other label options as well, including 6-carboxyfluorescein (FAM) and biotin. The double-FAM labeled LNA probes show similar staining intensities and signal-to-noise as the double-DIG labeled LNA probes. In the One-Day miRNA ISH Protocol, the FAM-labeled probes can therefore easily replace the DIG-labeled probes and have been implemented in the protocol as an alternative to the original DIG-based method (see Gould et al, Methods Mol Biol. 2017). Applicable in general, the concentration of the LNA probe (protocol step 4 on page 28) and the detecting antibody (protocol step 9 on page 30) should be optimized for best performance (for applications, see Nielsen et al, Methods Mol Biol 2014 and Knudsen et al, PlosOne 2015).

As described above, detection of the FAM label (or hapten) is performed with an alkaline phosphatase (AP)-conjugated anti-FAM antibody similarly as performed with the anti-DIG antibody used for detection of the DIG-labeled LNA probes. The detecting AP-conjugated anti-FAM antibody thus replaces the anti-DIG antibody, and is essentially the only modification in the miRNA ISH protocol.

LNA technology

Locked nucleic acids (LNA) are a class of high-affinity RNA analogs in which the ribose ring is "locked" in the ideal conformation for Watson-Crick binding. As a result, LNA oligonucleotides exhibit unprecedented thermal stability when hybridized to a complementary DNA or RNA strand. For each incorporated LNA monomer, the melting temperature (T_m) of the duplex increases by 2–8ºC. In addition, LNA oligonucleotides can be made shorter than traditional DNA or RNA oligonucleotides and still retain a high T_m. This is important when the oligonucleotide is used to detect small or highly similar targets.

Since LNA oligonucleotides typically consist of a mixture of LNA and DNA or RNA, it is possible to optimize the sensitivity and specificity by varying the LNA content of the oligonucleotide. Incorporation of LNA into oligonucleotides has been shown to improve sensitivity and specificity for many hybridization-based technologies including PCR, microarray and *in situ* hybridization.

Robust detection of all miRNA sequences, regardless of GC content.

The small sizes and widely varying GC content (5–95%) of miRNAs make them challenging to analyze using traditional methods. DNA- or RNA-based methods for miRNA analysis can introduce high uncertainty and low robustness, because the T_m of the oligonucleotide/miRNA duplex will vary greatly depending on the GC content of the sequences. This is especially problematic in applications such as microarray profiling and high-throughput experiments in which many miRNA targets are analyzed under the same experimental conditions.

Use of LNA-enhanced oligonucleotides overcomes these challenges. By simply varying the LNA content, oligonucleotides with specific duplex melting temperatures can be designed, regardless of the GC content of the miRNA. T_m-normalized primers, probes and inhibitors all perform well under the same experimental conditions.

Specific discrimination of highly similar targets

Another challenge of studying miRNAs is the high degree of similarity between the sequences. Some miRNA family members vary by only a single nucleotide. LNA can be used to enhance the discriminatory power of primers and probes to allow excellent discrimination of closely related miRNA sequences. LNA offers significant improvement in sensitivity and specificity and ensures optimal performance for all miRNA targets.

An LNA oligonucleotide offers substantially increased affinity for its complementary strand, compared to traditional DNA or RNA oligonucleotides. This results in unprecedented sensitivity and specificity and makes LNA oligonucleotides ideal for the detection of small or highly similar DNA or RNA targets.

Important Notes

Required probes and reagents

Table 1. Recommended control probes and reagents for the One-Day miRNA ISH Protocol.

Product	Cat. no.	Purpose
U6 snRNA, hsa/mmu/rno, miRCURY LNA miRNA Detection Probe, positive control, 5′-DIG labeled	Included in the miRCURY LNA miRNA ISH Buffer and Control (cat. no. 339459); also available separately	Initial optimization of protocol and assessment of sample quality
Positive Control Probe miRCURY LNA miRNA Detection Probe, 5′-DIG and 3′-DIG labeled	Select one of our wet-lab validated positive control probes (refer to Table 2)	Positive control miRNA known to be expressed in a cell type present in the tissue used. For verification of specific hybridization
Universal Negative Control Probe Scramble-miR, miRCURY LNA Detection Probe, negative control, 5′-DIG and 3′-DIG labeled	Included in the miRCURY LNA miRNA ISH Buffer and Control (cat. no. 339459); also available separately	Negative control to assess level of background signal from the probe chemistry (e.g., binding to scaffold proteins and cellular matrix)
miRCURY LNA miRNA Detection Probe, 5′-DIG and 3′-DIG labeled	Select a predesigned probe targeting your miRNA of interest (cat. nos. 339111, 339112) or order a custom designed miRNA probe (cat. nos. 339115, 339116)	Detection of your miRNA of interest; double-DIG labeling is recommended for increased sensitivity
miRCURY LNA miRNA ISH Buffer Set	339450	A formamide-free hybridization buffer developed specifically for miRCURY LNA miRNA Detection Probe-based ISH
Proteinase K Solution	Included in the miRCURY LNA miRNA ISH Buffer Set (cat. no. 339450) and miRCURY LNA miRNA ISH Optimization Kits (cat. nos. 339451–339459)	Demasks miRNAs, allowing probes to hybridize. Optimized treatment is essential to enable retention of the miRNA target

Proper controls and reliable reagents are crucial for optimal setup and performance of the ISH procedure steps and for ensuring successful results. Table 1 lists the required control probes and reagents for use with the One-Day miRNA ISH Protocol. The importance of each control is explained in more detail in the next section with examples in Table 2.

Optimizing the ISH procedure – the importance of controls

ISH is a powerful technique and the most common method for visualizing gene expression and localization in specific tissue and cell types. The technology is far from trivial and is often a very time-consuming and difficult procedure, requiring many steps of protocol optimization to achieve satisfactory ISH results. Detection of miRNA by conventional ISH analysis is no exception. The use of proper controls is crucial to limit the time spent on obtaining successful results.

LNA U6 snRNA control probe

The LNA U6 snRNA control probe stains rather easily if the protocol is followed and the sample is intact. Therefore, the signal can be used for the initial optimization of the protocol parameters by adjustment of Proteinase K treatment (concentration and duration) and the hybridization temperature (concentration and duration), as well as for testing the quality of the sample.

Positive miRNA control probe

The positive control miRNA miRCURY LNA Detection Probe is used as a control to verify that you are getting a good level of specific signal in the tissue type used. Table 2 shows a selection of miRNAs that have we have experimentally validated to be readily detectable in particular tissues, within a specific cell type. The positive miRNA control is important, as it readily shows that the signal you see is specific and limited to the relevant cells and cell types in the section. The positive miRNA control probe is also important for fine-tuning the Proteinase K treatment conditions. The U6 snRNA control probe will usually give a robust signal, even if Proteinase K treatment conditions are not fully optimized. Therefore, further fine-tuning might be needed when using the positive miRNA control probe and the actual miRNA probes of interest. See the Troubleshooting Guide for more information.

Negative scramble-miR control

The scramble-miR negative control probe is used once the Proteinase K treatment has been optimized. This control probe should give no signal. The scramble-miR negative control probe is designed to not have any targets, so any potential staining provides an indication of the level of background staining (noise). Further optimization might be needed to lower the background staining. For more information, see the suggestions about "Non-specific staining" in the Troubleshooting Guide.

Table 2. Selecting the appropriate positive control miRNA. The table displays the tissue(s) in which we have experimentally validated each of the miRNAs as a positive control, and in which cell type(s) the miRNA is typically detected. miRCURY LNA miRNA Detection Probes for each of these positive control miRNAs may be purchased separately, or as part of a miRCURY LNA miRNA ISH Optimization Kit.

Positive control miRNAs	hsa-miR-1-3p	hsa-miR-21-5p	hsa-miR-122-5p	hsa-miR-124-3p	hsa-miR-126-3p	hsa-miR-145-5p	hsa-miR-205-5p	hsa-miR-223-3p
Brain				✓				
Eye				✓	✓			
Muscle	✓				✓			
Lung					✓	✓		
Kidney					✓			
Liver			✓		✓			
Colon					✓	✓		✓
Cervix							✓	
Heart	✓				✓	✓		
Mammary gland					✓		✓	
Lung cancer		✓			✓	✓	✓	
Colorectal cancer		✓			✓	✓		
Breast cancer		✓			✓	✓	✓	
Kidney cancer		✓			✓	✓		
Cervix cancer		✓			✓	✓	✓	
Testis cancer					✓	✓		
Esophagus cancer								✓
Cell entity	Myocyte	Varies	Hepato-cyte	Neuron	Endo-thelial	Smooth muscle	Basal cells	Granulo-cyte
Product. number. (for purchasing the miRCURY LNA miRNA Detection Probe separately)	YD00 619868-xxx	YD00 619870-xxx	YD00 619864-xxx	YD00 619867-xxx	YD00 619866-xxx	YD00 619865-xxx	YD00 619873-xxx	YD00 619871-xxx
Cat. no. for miRCURY LNA miRNA ISH Optimization Kit	339451 Kit 1	339452 Kit 2	339453 Kit 3	339454 Kit 4	339455 Kit 5	339457 Kit 7	339458 Kit 8	339459 Kit 9

Equipment and Reagents to be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs) available from the product supplier.

Protocol and equipment considerations

ISH protocols vary extensively due to different equipment setup and laboratory routines. The One-Day miRNA ISH Protocol details the process of manual ISH on formalin-fixed and paraffin embedded tissue samples using double-DIG or double-FAM labeled miRCURY LNA miRNA Detection Probes. For the ISH steps, we recommend a hybridization station that allows precise and rapid temperature adjustments, such as the Dako Hybridizer. The One-Day miRNA ISH Protocol was developed using a hybridization station, but if one is not available, conventional hybridization ovens may be used (see "Can I use a hybridization oven instead of a Dako Hybridizer?" on page 34).

For the immunohistochemical steps, we have had good experience with both horizontal humidifying chambers and Shandon™ Sequenza slide rack systems. The chromogenic ISH assay is based on the use of DIG- or FAM-labeled probes and therefore requires proper detection reagents, such as alkaline phosphatase-conjugated anti-DIG and NBT-BCIP substrate for DIG-labeled probes.

In addition to the miRCURY LNA miRNA Detection Probes and the miRCURY LNA miRNA ISH Buffer Set (FFPE), various reagents and equipment are needed to perform the ISH experiments as described in the protocol. The list below includes recommendations for specific products that have been shown to work well with the One-Day miRNA ISH Protocol. Refer to section "Preparation of reagents and buffers" for detailed instructions on preparing the buffers and reagents.

Reagents and equipment:

- For DIG-labeled probes: Sheep anti-DIG-AP from Roche (Sigma-Aldrich, cat. no. 11093274910)
- For FAM-labeled probes: Sheep anti-fluorescein-AP from Roche (Sigma-Aldrich, cat. no. 11426338910)
- Sheep serum (Jackson Immunoresearch, cat. no. 013-000-121)
- NBT/BCIP ready-to-use tablets (Roche, cat. no. 11 697 471 001) or equivalent
- Levamisole (Fluka, cat. no. 31742 or equivalent)
- Syringe and Whatman™ 1001–125 filter paper for filtering nuclear counterstaining solution
- Nuclear Fast Red nuclear counterstain (Vector laboratories, cat. no. H-3403) or equivalent
- Mounting medium, Eukitt® (VWR, cat. no. 361894G)
- RNaseZap® (Ambion)
- Hybridizer, such as Dako Hybridizer, Vysis ThermoBrite or Invitrogen Spotlight Hybridizer
- Humidifying chamber or equivalent for immunohistochemical detection
- Superfrost™ Plus slides
- Coverslips
- Slide rack(s) and several glass jars for deparaffinization, dehydration and washes
- Xylene (for deparaffinization)
- Ethanol (for hydration and dehydration)
- PBS, sterile
- SSC buffer, ultrapure
- 1 M Tris-HCl, pH 7.4
- 0.5 M EDTA

- 5 M NaCl
- Tween-20 (Sigma, cat. no. P1379)
- 30% BSA (Sigma, cat. no. A9576)
- For KTBT buffer: Tris-HCl, NaCl and KCl (see Table 4 on page 24)
- Dako Pen (or equivalent hydrophobic PAP pen)
- Non-stick, RNase-free microcentrifuge tubes
- Sterile filter tips

Preparing for the ISH Protocol

Preparing and aliquoting DIG- and FAM-labeled LNA probes before first use

The following options are possible for aliquoting and storing the miRCURY LNA miRNA Detection Probes:

1. The probes may be stored at 2–8°C if they will be used within 4 weeks.
2. For longer storage, divide the miRCURY LNA miRNA Detection Probes into 5 µL or 10 µL aliquots in non-stick, RNase-free tubes and store at –15 to –30°C or below. Avoid multiple freeze-thaw cycles.
3. If you have already determined the optimal probe concentration, you can prepare pre-diluted probe aliquots in miRNA ISH Buffer and store at –15 to –30°C or below. For details on making 1x miRNA ISH Buffer and how to denature and dilute the probes, refer to Table 5 on page 24. On the day of use, thaw the pre-diluted probe to room temperature and apply directly to the sections.
   Note: The probe concentration must be optimized. Refer to "Identifying the ISH sensitivity level" on page 40.

Checking that equipment and reagents are ready for use

Before starting the experiment, we recommend that you follow the steps below to ensure that all required lab equipment and reagents for the miRNA ISH procedure are available and ready for use.

1. Prepare and store miRCURY LNA miRNA Detection Probes and ISH Buffer as described in "Preparing and aliquoting DIG- and FAM-labeled LNA probes before first use" on page 19.
2. The Proteinase K is already in solution and should be diluted immediately before use, as described in Table 5 on page 24.
3. Establish histology lab environment for RNA work (refer to pages 21–21).
4. Prepare samples, including fixation of tissue and FFPE preparations, as described in "FFPE sample requirements and comments on tissue fixation" on page 21.
5. Cut FFPE sections according to the "Tissue-sectioning guidelines" on page 38.
6. Prepare reagents and buffers that can be made ahead of time as described in "Preparation of reagents and buffers" on page 22. Note that the reagents described in Table 5 must be prepared on the day of the miRNA ISH procedure.
7. Review and understand the steps of the "Procedure: One-Day miRNA ISH Protocol" on page 27.
8. Evaluate the appropriate Proteinase K treatment range for each sample type. Refer to "Determining the appropriate Proteinase K treatment range" on page 39.
9. Determine the ISH sensitivity level using the LNA U6 snRNA probe. It may be necessary to repeat steps 7 and 8 to gradually improve the performance of the protocol. Refer to "Identifying the ISH sensitivity level" on page 40.
10. Run the miRNA ISH protocol with the miRCURY LNA miRNA Detection Probe and the LNA Scramble-miR probe.
11. Optimize the hybridization temperature and probe concentration for each probe.

An overview of the workflow for the One-Day miRNA ISH Protocol is shown on page 26.

Preventing RNase contamination

RNA work requires specific handling and precautions to prevent RNase contamination of the reagents and degradation of the RNA sample. Every step in the miRNA ISH procedure, including tissue sectioning and DIG detection, must take place in a clean and nuclease-free environment. We recommend that all surfaces are cleaned with RNaseZap, RNase AWAY® or other RNase removal solution. Wear gloves during the entire process, and only use RNase-decontaminated glassware. All buffers and reagents should be prepared using RNase-free water only, such as RNase-grade Milli-Q® water or DEPC-treated water, and should be autoclaved if specified in the preparation instructions.

FFPE sample requirements and comments on tissue fixation

Like other fragile RNA molecules, miRNAs are sensitive to degradation. Therefore, fast and sufficient fixation of tissue specimens is important for successful ISH analyses. For ISH analysis of human specimens, standard overnight fixation in neutral-buffered formalin, followed by paraffin-embedding often works well. For mouse tissues, perfusion fixation with 4% fresh PFA is recommended before standard overnight fixation in formalin. Consult appropriate animal care guidelines before setting up this protocol, such as: *The Laboratory Mouse* by Mark A. Suckow, Peggy Danneman, Cory Brayton (CRC Press) or *Pathology of Genetically Engineered Mice* by Jerrold Michael Ward, Joel F. Mahler, Robert R. Maronpot (Iowa State University Press). Optimization of the assay performance should preferably be based on analysis of at least four FFPE blocks. For detailed guidelines on tissue sectioning, refer to "Tissue-sectioning guidelines" on page 38.

Glassware

To reduce potential RNase contamination of glassware, it is recommended to autoclave all glassware or to heat-treat all glassware for 8 hours at 180°C. Prior to the heat treatment, it is recommended to wrap all items, including appropriate stacks of coverslips, in aluminum foil. Keep treated glassware separate from untreated glassware.

Preparation of reagents and buffers

In addition to the hybridization buffer and Proteinase K buffer supplied with the miRCURY LNA miRNA ISH Buffer Set, a number of other reagents, buffers and materials must be prepared prior to initiating the miRNA ISH experiment. Refer to page 16 for a list of recommended materials. Recipes for preparing the required reagents and buffers are listed in Tables 3–5.

Important: Note that many of the reagents should be freshly prepared on the day of the experiment, or even immediately before use.

Table 3. Reagents needed for the ISH procedure.

Reagent	Details
Antibody blocking solution	PBS, 0.1% Tween, 2% Sheep serum, 1% BSA, (see table 3 for details)
Antibody dilutant solution	PBS, 0.05% Tween, 1% sheep serum, 1% BSA (see table 3 for details)
Sheep anti-DIG-AP or anti-FAM-AP	See table 3
NBT/BCIP ready-to-use tablets	See table 3
Levamisole	For blocking endogenous AP activity. Prepare a 100 mM stock
Nuclear Fast Red	Nuclear counterstain

Table 4. Buffers and stocks to prepare and autoclave* prior to the miRNA ISH procedure.

Buffer	Preparation instructions
Proteinase K buffer	To 900 ml RNase-free water, add: 5 ml of 1 M Tris-HCl, pH 7.4 2 ml of 0.5 M EDTA 0.2 ml of 5 M NaCl Adjust volume to 1000 ml and autoclave*
20x SSC, pH 7.0.	If purchased as RNase-free, then use as-is.
SSC solutions	5x SSC: 250 ml 20x SSC + 750 ml water 1x SSC: 50 ml 20x SSC + 950 ml water 0.2x SSC: 10 ml 20x SSC + 990 ml water Autoclave
PBS-T 0.1%, pH 7.4	Add 1 ml of Tween-20 to 1 liter of PBS and autoclave*
KTBT (AP stop solution)	To 900 ml RNase-free water, add: 7.9 g Tris-HCl (50 mM final concentration) 8.7 g NaCl (150 mM final concentration) 0.75 g KCl (10 mM final concentration) Adjust volume to 1000 ml. Do not adjust pH. Autoclave*.

* Autoclave buffers as indicated to minimize RNase activity. The RNaseAlert® Lab test Kit (Ambion) is an easy and fast test that is recommended for optional testing for potential RNase activity in buffers and reagents.

Table 5. Reagents that must be prepared on the day of the miRNA ISH procedure.

Reagent	Preparation instructions			
Proteinase K reagent (1x)	Prepare immediately before use. To prepare the 1x Proteinase K reagent, add 7.5 µl of the Proteinase K Solution to 10 ml Proteinase K buffer (prepared in Table 4). See further recommendations in "Determining the appropriate Proteinase K treatment range" on page 39.			
Hybridization mix (miRNA ISH buffer and LNA Detection Probes)	1. Dilute the 2x miRNA ISH buffer 1:1 with RNase-free water. For example, mix 1 ml of 2x miRNA ISH buffer with 1 ml RNase-free water to give 2 ml 1x miRNA ISH buffer. 2. For each probe to be used in the experiment, place the volume of LNA probe indicated below into a 2 ml non-stick, RNase-free tube. 3. Denature the probes at 90°C for 4 minutes. 4. Place the tubes into a microcentrifuge and spin down briefly. 5. Immediately add the 2 ml of 1x miRNA ISH buffer to each of the tubes containing the different LNA probes.			
Probe	**Final probe concentration**	**Probe volume**	**Dilution factor**	**1x miRNA ISH Buffer volume**
LNA U6 snRNA (0.5 µM)	1 nM	4 µl	1:500	2 ml
LNA miRNA probe (25 µM)*	20 nM	1.6 µl	1:1250	2 ml
LNA miRNA probe (25 µM)*	40 nM	3.2 µl	1:625	2 ml
LNA Scramble-miR probe (25 µM)	40 nM	3.2 µl	1:625	2 ml
Antibody blocking and dilutant solutions	1. To make 10 ml blocking and 10 ml dilutant solution, start with 15 ml PBS-T (prepared in Table 4) and add 300 µl sheep serum (2% final concentration). Label the tube "Blocking solution". 2. Remove 5 ml from the tube in Step 1, place into a new tube and label "Dilutant solution". 3. To the tube labeled "Blocking solution", add 330 µl 30% BSA, for a final concentration of 1%. The blocking solution is now ready to use. 4. To the tube labeled "Dilutant solution", add 5 ml PBS and 330 µl 30% BSA, for a final concentration of 0.05% Tween, 1% sheep serum, and 1% BSA. The Dilutant solution is now ready for use.			
Anti-DIG or anti-FAM reagent	Dilute the sheep anti-DIG-AP or anti-FAM-AP antibody 1:800 in Antibody Dilutant solution (see above). The dilution range should be 1:500–1:2000.			

Reagent	Preparation instructions
AP substrate	Immediately prior to use, dissolve a NBT-BCIP tablet in Milli-Q water according to the manufacturer's instructions. Add Levamisol to a final concentration of 0.2 mM. Example: For preparing 10 ml AP substrate, add 20 µl Levamisol stock. Protect from light before and during incubation.

* Suggested starting concentrations for miRNA probes can be found in Table 11 on page 41.

Left-over hybridization mix can be stored at –15 to –30°C and will be stable for up to 6 months. Avoid multiple freeze-thaw cycles.

Workflow overview for the One-Day miRNA ISH Protocol

Below is an overview of the workflow for the miRNA ISH protocol for FFPE samples. The numbers refer to the particular protocol steps. See details in the "Procedure: One-Day miRNA ISH Protocol", on page 27. The workflow can be followed for both the initial protocol optimization with the LNA probes provided with the kit as well as the subsequent specific miRNA detection using the miRCURY LNA miRNA Detection Probes.

Table 6. One-Day miRNA ISH Protocol workflow overview.

Step	Process*	Equipment	Time	Temperature
1	Deparaffinization	Side rack and jars	40 min	Room temperature (15–25ºC)
2	Proteinase K incubation	Hybridizer	10 min	37ºC
4	*In situ* hybridization	Hybridizer	60 min	50–60ºC
6	Stringent washes	Water bath	30 min	50–60ºC
8	Blocking	IHC staining racks	15 min	Room temperature (15–25ºC)
9	Anti-DIG-AP	IHC staining racks	60 min	Room temperature (15–25ºC)
11	AP reaction	IHC staining racks in oven	120 min	30ºC
14	Counterstaining	IHC staining racks	10 min	Room temperature (15–25ºC)
16	Dehydration	Side rack and jars	10 min	Room temperature (15–25ºC)
17	Mounting		5 min	Room temperature (15–25ºC)

* PBS washing steps are excluded from this overview. Total time required is 7 hours.

Procedure: One-Day miRNA ISH Protocol

Important notes before starting

This protocol describes every step in the miRNA ISH analysis. When setting up the miRNA ISH experiment for the first time, we recommend that you follow all of the procedures described in "Preparing for the ISH Protocol" on page 19, as well as the three optimization steps below.

Optimization

1. First, optimize the protocol parameters with the LNA U6 snRNA positive control probe by adjusting the hybridization temperature and Proteinase K treatment.
2. Conduct a control study using the optimized protocol parameters with the double-DIG or double-FAM LNA miRNA positive and negative control probes. Adjust the hybridization temperature and Proteinase K treatment to obtain a strong, specific miRNA ISH signal.
3. Finally, detect the miRNA(s) of interest using the appropriate miRCURY LNA miRNA Detection Probe(s) with the defined protocol parameters, keeping in mind that the hybridization temperature may need to be adjusted.

ISH procedure

1. Deparaffinization: Deparaffinize slides in xylene and ethanol solutions at room temperature (15–25ºC) by placing the slides with sections into a slide rack and transferring the rack from glass jar to glass jar according to Table 7, ending up in PBS.

Table 7. Deparaffinization steps.

Deparaffinization step	Solvent	Duration
1	Xylene	5 min
2	Xylene	5 min
3	Xylene	5 min
4	99.9% ethanol	Immerse 10 times
5	99.9% ethanol	Immerse 10 times
6	99.9% ethanol	5 min
7	96% ethanol	Immerse 10 times
8	96% ethanol	5 min
9	70% ethanol	Immerse 10 times
10	70% ethanol	5 min
11	PBS	2–5 min

2. Proteinase K incubation: Immediately before use, add Proteinase K to Proteinase K buffer as described in Tables 4–5 on pages 23–24. Place the slides onto a flat surface and apply approximately 300 µl of Proteinase K reagent to each slide, fully covering the section. Incubate slides for 10 min at 37°C (e.g., in a Dako Hybridizer).

   Note: If using a hybridizer, remove the humidity control strips.

   Note: The Proteinase K concentration range must be optimized for individual tissues (see "Determining the appropriate Proteinase K treatment range" on page 39).

3. Wash: Place the slides into a slide rack inside a jar containing PBS. Wash twice in PBS.
4. Hybridization: Place the slides onto a flat surface and apply 50 µl of hybridization mix, as prepared in Table 5 on page 24. Avoid touching the tissue sections with the pipette

tip. Then apply a sterile coverslip to each section, carefully avoiding air bubbles. Place the slides into the hybridizer (equipped with humidity control strips with Milli-Q water), and start a hybridization program for 1 hour. The hybridization temperature must be optimized for individual probes.

Note: The probe concentrations will need to be optimized for optimal miRNA ISH signal. See "Identifying the ISH sensitivity level" on page 40. For the initial protocol optimization, the probe concentrations could be:

a) 1 nM LNA U6 snRNA probe

b) 40 nM double-DIG or double-FAM LNA miRNA probe

Note: For guidance on optimizing the hybridization temperature, see "Identifying the optimal hybridization temperature" on page 42.

5. Disassembly: Prepare a jar containing 5x SSC at room temperature (15–25ºC). One at a time, carefully remove the coverslip and immediately place the slide into a slide rack in the 5x SSC buffer.

   Note: If the coverslips do not easily detach, place the slides directly into the 5x SSC buffer. After a few minutes, the coverslips will detach. Then place the slide without the coverslip into the jar containing 5x SSC.

6. Stringent washes: Wash the slides in glass jars according to the steps in Table 8. To ensure sufficient stringency, perform the washes in glass jars placed in a water bath set to the hybridization temperature.

Table 8. SSC wash steps.

SSC wash step	Buffer	Duration	Temperature
1	5x SSC	5 min	Hybridization temp
2	1x SSC	5 min	Hybridization temp
3	1x SSC	5 min	Hybridization temp
4	0.2x SSC	5 min	Hybridization temp
5	0.2x SSC	5 min	Hybridization temp
6	0.2x SSC	5 min	Room temperature (15–25ºC)

7. Apply hydrophobic barrier: Transfer the slides to glass jars containing PBS. Remove each slide and apply a hydrophobic barrier around the tissue sections using a Dako Pen, following the manufacturer's instructions. Alternatively, if you are using Shandon Slide Racks, then assemble the slides on coverplates using PBS-T.

   Important: Do not allow the tissue sections to dry out during this step or during the subsequent immunohistochemistry steps.

8. Blocking: Place the slides into a humidified chamber and incubate with blocking solution for 15 min at room temperature (15–25ºC).

   Important: Carry out steps 8–15 in the humidifying chamber or in Shandon Slide Racks.

9. Anti-DIG or anti-FAM: Remove the blocking solution and apply the anti-DIG or anti-FAM reagent, as prepared in Table 5 on page 24, depending on your LNA probe label. Incubate for 60 min at room temperature (15–25ºC).

10. Wash: Wash the slides with PBS-T, 3 times for 3 minutes each.

11. AP reaction: Apply the freshly prepared AP substrate to the sections (see Table 5 on page 24) and incubate the slides for 2 H at 30°C in the humidifying chamber. Protect from light during the development.

12. KTBT incubation: Incubate the slides in KTBT buffer 2 times for 5 min each to stop the reaction.

13. Wash: Wash the slides with water, 2 times for 1 min each.

14. Counterstaining: Depending on the size of the tissue, apply 200–300 µl Nuclear Fast Red nuclear counterstain for 1 minute.

    Note: Immediately before application, we recommend filtering the Nuclear Fast Red solution through a paper filter (e.g., Whatman 1.001–125) to remove undissolved color precipitates.

15. Rinse: Remove the slides from the humidifying chamber and place them into a slide rack in a glass jar containing tap water. Carefully rinse the slides with running tap water for approximately 10 min.

16. Dehydration. Dehydrate the slides in ethanol solutions according to the steps in Table 9. Place the slides onto clean paper towels after the final step.

Table 9. Ethanol dehydration steps.

Ethanol dehydration step	Solvent	Duration
1	70% ethanol	Immerse 10 times
2	70% ethanol	1 min
3	96% ethanol	Immerse 10 times
4	96% ethanol	1 min
5	99.9% ethanol	Immerse 10 times
6	99.9% ethanol	1 min

17. Mounting: Mount the slides directly using 1–2 drops of mounting medium (e.g., Eukitt). Do not let the sections dry out during this step.
18. Microscopy: Allow the precipitate to settle overnight and analyze the slides by light microscopy the next day.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and/or protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

Issue	Comments and suggestions
No signal	If no signal is obtained with the LNA U6 snRNA probe incubated at 10 nM, ensure that all reagents were prepared according to the recommendations and are RNase free (see "Preparation of reagents and buffers" on page 22). Always test sections from more than one block; a minimum of four sections is recommended.
Insufficient sensitivity level with the LNA U6 snRNA probe	The LNA U6 snRNA signal should be intense when incubated in the range of 0.1–1 nM. If this is not the case, ensure that the buffers have been prepared correctly (see "Preparation of reagents and buffers" on page 22) and that the tissue sections are 5–7 µm thick. Ensure that the AP reaction takes place at 30°C. Low sensitivity may also be caused by RNase contamination during sectioning or handling during the ISH protocol. Make sure that all steps of the ISH protocol are performed in an RNase-free environment. Be aware that both insufficient and hard fixation of tissue samples may result in a low signal. Therefore, it is necessary to test several blocks in parallel and avoid drawing conclusions from a single sample.
Strong U6 snRNA signal but no or low miRNA signal	If a strong U6 snRNA signal is obtained with 0.1–0.5 nM probe, but no signal is obtained with the supplied positive control LNA miRNA probe, it is most likely due to sub-optimal Proteinase K treatment. Hence, the Proteinase K concentration or duration of treatment should be optimized (see "Determining the appropriate Proteinase K treatment range" on page 39). To boost a weak signal, remove the anti-DIG reagent (protocol step 9 on page 30) halfway through the incubation (e.g., after 30 minutes) and apply new, unused reagent for the second half of the incubation. The same approach can also be used for the AP substrate incubation in protocol step 11. For low copy number targets, it may be possible to increase the signal with Tyramide Signal Amplification (TSA)-based systems.

Issue	Comments and suggestions
Non-specific staining	It is necessary to determine whether non-specific staining obtained with the LNA Scramble-miR probe is related to the DIG-labeled probe itself, the detecting antibody or to endogenous enzymatic reactions. This can be done using a systematic approach: test the effect of excluding individual reagents including the DIG-labeled probe, the AP-conjugated anti-DIG or both. If staining is obtained in the absence of AP-conjugated anti-DIG, then endogenous AP is present. If staining is obtained in the absence of the DIG-labeled probe (and no endogenous AP activity is observed), then staining is related to the detecting antibody. If abundant, endogenous enzymatic reactivity (e.g., in some intestinal areas and placenta) cannot be prevented by Levamisol, it may require changing to another detection approach, such as TSA-based fluorescence (see Nielsen and Holmstrøm, Methods Mol. Biol., 2013). Some non-specific staining can be caused by improperly maintained SSC wash buffer temperatures. It is important to ensure that the SSC wash buffers are preheated to and maintained at the hybridization temperature (see protocol step 6 on page 29).
High background staining	If all possibilities for non-specific staining mentioned above have been ruled out and if the specific signal from the miRNA probe is strong, high background signal can often be reduced by increasing the hybridization temperature and/or increasing the duration of the stringency washes.
Non-specific staining of ECM	Non-specific staining of extracellular matrix (ECM) may occur if the concentration of the detecting antibody is too high.
Sections fall off after de-paraffinization	Avoid storing paraffin sections at –20°C. Small, thick sections fall off more easily than large, thin sections. Ensure that the glass slides you are using have electrostatic properties (e.g., SuperFrost Plus slides). When transferring sections from the water bath to slides, it is important to let all excess water drain/evaporate from the section and slide to avoid trapping water or air bubbles under the section. For fatty tissues or loose connective tissue (e.g., tissue from normal breast), increasing the duration of the melting step from 45 minutes to 60 minutes sometimes helps.

Frequently asked questions

Can I use a hybridization oven instead of a Dako Hybridizer?

When using a hybridization oven during the hybridization step, it may be advisable to seal the coverslips using Fixogum. The slides can be placed as such into the hybridization oven without humidifying conditions. However, humidifying conditions may be tried, e.g., by using 1x SSC buffer. To establish a more stable hybridization temperature, place a metallic plate, such as the inserts from a multiblock heater, into the oven. Place the slides onto the plate and hybridize for 1–2 hours. Then proceed with protocol step 5 on page 29.

Can I use the ISH protocol for fresh frozen tissue?

The protocol can be adapted to cryo sections (see Nielsen et al., Methods Mol. Biol. 2014). The protocol will require optimization for individual sample types and miRNA targets.

Can I pause the ISH procedure?

The individual steps in the One-Day miRNA ISH Protocol have been optimized to accommodate a one-day protocol. The PBS steps may be prolonged, but we do not recommend extending the protocol to more than one day.

What happens if sections dry out?

Sections should be maintained in buffered solutions after the hybridization step. Drying out of tissue sections may cause protein denaturation, which may be particularly harmful to the detecting antibody and its conjugated alkaline phosphatase. This may lower the sensitivity of the assay significantly and may also cause background staining. Drying out of tissue sections may also reduce the quality of the tissue morphology.

Can I use other detection methods?

The DIG-labeled LNA probes can be detected using alternative methods for DIG detection, such as TSA-based systems. Using alternative detection systems may necessitate adding steps at various stages of the protocol and requires optimization.

Can I detect two different miRNAs in the same section?

Two miRNAs can be detected in the same FFPE section by double-fluorescence analysis. It may also be possible using double-chromogen detection; however, this has not been documented. The double-miRNA staining assay requires two miRCURY LNA miRNA Detection Probes with different labels (haptens), such as one LNA probe double-labeled with DIG and one LNA probe double-labeled with FAM, or alternatively, DIG and Biotin labels (see more details on page 36 under "Can I use other types of double labeling as an alternative to double-DIG?"). Fluorescence detection of miRNAs is performed using peroxidase-conjugated sheep antibodies and tyramine substrates (TSA), such as TSA-Cy3 (red fluorophore) and TSA-FITC (green fluorophore). ISH can be performed by mixing the two probes. However, if different hybridization temperatures are required for optimal hybridization, or if the two probes interact, hybridization should be performed consecutively. After the two probes have hybridized, one of them is detected with a peroxidase-conjugated antibody (e.g., anti-DIG) followed by incubation with an appropriate TSA substrate (e.g., TSA-FITC). After a peroxidase blocking step (in 3% H_2O_2), the other probe is detected with the proper peroxidase-conjugated antibody (e.g., anti-FAM), followed by another TSA substrate (e.g., TSA-Cy3). All steps need optimization, including stringent washes and washes in PBS after antibody incubations. The staining intensity should be significant and should be evaluated against negative control sections to adjust for background staining and autofluorescence. The slides can be mounted with DAPI mounting medium.

In the study by Sempere et al. (Clin. Cancer Res. 2010), miR-205 was co-detected with U6 snRNA using double-FAM and double-biotin labeled miRCURY LNA miRNA Detection Probes, respectively. miR-205 and U6 snRNA signals were revealed by sequential TSA

reactions with TSA-FITC (green for miR-205 probe) and TSA-rhodamine (red for U6 snRNA probe) substrates.

Can I detect both miRNA and protein in the same section?

An miRNA can be detected together with a protein in tissue sections by combined ISH and immunohistochemistry double-fluorescence staining. The main limitation in such an assay is the compatibility of pretreatment procedures required for the miRNA probe and the primary antibody. miRNA ISH is limited to protease-dependent pretreatment, and therefore, some literature searches may be needed to identify an appropriate primary antibody that works well with proteolytic pretreatment. It is recommended to evaluate the performance of the antibody prior to use in the ISH study. Alternative strategies may involve cryo sections, in which miRNA ISH can be performed (Nielsen et al., Methods Mol. Biol., 2014) and most primary antibodies can be applied without pretreatment.

For the combined miRNA ISH and immunohistochemistry, the miRNA ISH is performed first, essentially as described in this protocol. The probe is detected with a peroxidase-conjugated antibody (e.g., anti-DIG) followed by an appropriate TSA substrate (e.g., TSA-FITC). After stringent washes, the sections are incubated with the primary antibody (e.g., rabbit-anti-cytokeratin), followed by a proper fluorophore-conjugated antibody (e.g., Cy3-conjugated anti rabbit). The One-Day miRNA ISH Protocol using miRCURY LNA miRNA Detection Probes has been described in detail by Nielsen and Holmstrøm (Methods Mol. Bio., 2013). The slides can be mounted with DAPI mounting medium.

Can I use other types of double labeling as an alternative to double-DIG?

The original One-Day miRNA ISH Protocol was developed using DIG-labeled miRCURY LNA miRNA Detection Probes (Jorgensen et al, Methods, 2010). However, the miRCURY LNA miRNA Detection Probes can be obtained with other labels as well, including FAM and biotin. For these probes, detection of the anti-DIG antibody is replaced by detection of AP-conjugated antibodies (anti-FAM or anti-biotin), or streptavidin for biotin. The double-FAM labeled LNA probes show the same signal-to-noise ratio as the double-DIG labeled LNA

probes and can be used equally well. Thus, the One-Day miRNA ISH Protocol can easily be adapted for use with FAM-labeled LNA, as it is also described in the present protocol, in which FAM-labeled LNA probes are introduced as an equally interchangeable alternative to double-DIG labeled LNA probes. The concentration of the miRCURY LNA miRNA Detection Probe and the detecting antibodies should always be optimized for best performance, regardless the choice of label type.

The use of FAM-labeled probes in miRNA ISH introduces new and interesting ISH applications. The carboxyfluorescein moiety (FAM) is a fluorophore like fluorescein isothiocyanate (FITC). The fluorescence emission from a double-FAM labeled LNA probe is not sufficient for visualizing the bound probe itself in standard epifluorescence microscopy. However, the double-FAM labeled LNA probes have at least two valuable applications. First, because antibodies can be raised against the FAM molecule, double-FAM labeled LNA probes can replace double-DIG labeled LNA probes in the standard miRNA ISH protocol, as described above. Secondly, the double-FAM labeled LNA probes can be used in double-ISH assays, in which one miRNA is detected with a double-FAM labeled LNA probe, and the other miRNA is detected with a double-DIG labeled LNA probe. A derived application thereof is the combined ISH detection of two different RNA targets performed with the two differently labeled LNA probes, respectively.

Appendix: Tips for the One-Day miRNA ISH Protocol

Tissue-sectioning guidelines

It is strongly recommended that you wear gloves during paraffin sectioning and maintain an RNase-free environment during all downstream procedures. Use only heat-treated glassware and RNase-free water. Use SuperFrost Plus slides taken directly from new packages.

Workstation and microtome

Before starting with tissue sectioning, clean the entire workstation (bench top, microtome, blade holder, brushes, tweezers, cooling plate, water bath, etc.) with RNase Zap or RNase Away.

Cutting sections

1. Prepare a water bath with room temperature (15–25ºC) RNase-free water and a warm-water bath with RNase-free water at 40–50°C (depending on the paraffin type).
2. Insert a new, disposable blade into the knife carrier and place the paraffin block into the cassette clamp. Trim the block to avoid the first couple of sections. It is recommended to cool the FFPE blocks on a cooling plate to approximately –15°C before cutting, to better control the section thickness.
3. Cut 6 µm thick paraffin sections and place them into the room temperature (15–25ºC) RNase-free water, so that folding can be reversed. Transfer the sections to the heated water bath, and allow the tissue sections to stretch briefly. It is recommended to mount the sections immediately thereafter onto electrostatic-treated slides, such as SuperFrost Plus slides, taken directly from a new, non-contaminated package.

4. Let the paraffin sections dry for 1–2 H at room temperature (15–25ºC). They can be stored at 2–8°C for up to one week. Avoid melting the paraffin until the day prior to the ISH analysis.
5. Melt the paraffin in a 60°C oven for 45 min on the day before conducting the ISH experiment. Store the slides overnight at 2–8°C in an RNase-free environment.

Determining the appropriate Proteinase K treatment range

The degree of Proteinase K treatment depends on fixation and tissue of origin. In general, the harder the fixation, the more Proteinase K is needed; however, there are lower and upper limits. For the Proteinase K treatment step, we recommend that you vary the concentration or the duration, as indicated in Table 10. Optimal starting values are indicated in parenthesis after the recommended testing ranges.

To identify the optimal Proteinase K conditions, start by testing the LNA U6 snRNA probe at 4–5 different concentrations between 0.1–2.0 nM, using the One-Day miRNA ISH Protocol. Once the optimal conditions have been established, start testing the double-DIG labeled LNA miRNA probe (positive miRNA control) and the LNA Scramble-miR probe (negative control).

Table 10. Optimization of Proteinase K treatment.

	Fixation method	Temperature	Proteinase K reagent amount*	Treatment duration
Adjust the concentration of Proteinase K:				
Human FFPE	Routine formalin	37ºC	0.33–2x Proteinase K reagent	10 min
Mouse FFPE	PFA perfusion	37ºC	0.03–0.33x Proteinase K reagent	10 min
Adjust the duration of Proteinase K treatment:				
Human FFPE	Routine formalin	37ºC	1x Proteinase K reagent	5–30 min (10 min)
Mouse FFPE	PFA perfusion	37ºC	0.13x Proteinase K reagent	5–30 min (10 min)

* Preparation of 1x Proteinase K reagent is described in Table 5 on page 24. To prepare 2x Proteinase K reagent, for example, use double the amount of Proteinase K Solution specified in the table. To prepare 0.33x Proteinase K reagent, use 1/3 of the amount of Proteinase K Solution specified.

Identifying the ISH sensitivity level

To identify the sensitivity of the performance of the ISH protocol, we recommend that you prepare dilutions of the LNA U6 snRNA probe. Figure 3 shows that the LNA U6 snRNA probe should provide a significant ISH signal at a concentration between 0.03–0.3 nM. The U6 snRNA ISH signal should be intense (as shown for the 0.1 nM concentration in Figure 3), when the probe is incubated at 0.1–2.0 nM before moving on with the double-DIG probes for miRNA ISH.

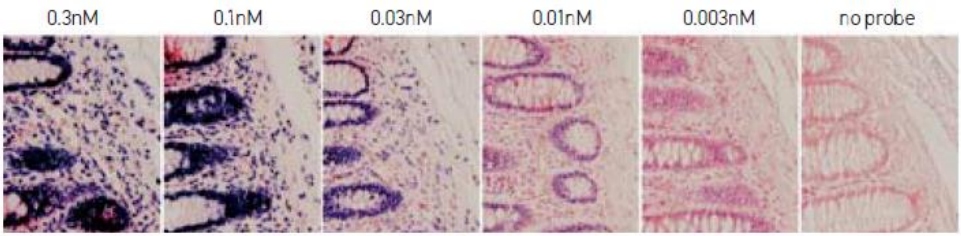


Figure 3. Optimization of the detection signal for the U6 snRNA positive control probe. On consecutive sections from FFPE tissue sample from normal human colon, the hybridization conditions are optimized by applying increasing concentrations of the U6 snRNA positive control probe.

Once the optimal hybridization conditions are determined for the LNA U6 snRNA probe, we recommend that you use the suggested starting concentrations in Table 11 for the double-DIG labeled miRCURY LNA miRNA Detection Probes, which are also supplied in the miRNA ISH Optimization Kits. The optimal hybridization temperature is in the 50–60°C range).

Table 11. miRCURY LNA miRNA Detection probe, suggested concentrations.* Typical positive miRNA controls are also available in the miRCURY LNA miRNA ISH Optimization Kits.

Probe name	Concentration, nM	RNA T_m
hsa-miR-1-3p	20	81°C
hsa-miR-21-5p	20–40	83°C
hsa-miR-122-5p	40	85°C
hsa-miR-124-3p	40	90°C
hsa-miR-126-3p	40	84°C
hsa-miR-145-5p	20	84°C
hsa-miR-205-5p	20–40	87°C
hsa-miR-223-3p	40	83°C

* The optimization range for double-DIG or double-FAM labeled LNA probes could be between 20–80 nM.

Identifying the optimal hybridization temperature

Optimal performance of an ISH probe is related to its signal-to-noise ratio. Oligonucleotide probes, and especially LNA containing probes, can potentially hybridize to highly similar sequences if the hybridization temperature is too low. The LNA probes typically result in a high signal-to-noise ratio at around 55°C using the One-Day miRNA ISH Protocol. The positive control LNA probes supplied with the miRNA ISH Optimization Kits typically result in a high signal-to-noise ratio at 55°C using the One-Day miRNA ISH Protocol. The LNA probes also hybridize at 60°C, but generally provide weaker signals. At 50°C, the LNA probes give stronger signals, but the risk of cross-hybridization to highly similar sequences (in RNA transcripts or the genome) will increase at low hybridization temperatures. As a rule-of-thumb, hybridization should be performed at 30°C below the given RNA T_m (or 20°C below DNA T_m).

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Ordering Information

Product	Contents	Cat. no.
miRCURY LNA miRNA Detection Probe (1 nmol)	1 nmol oligonucleotide, dried down in tube format	339111
miRCURY LNA miRNA Detection Probe (10 nmol)	10 nmol oligonucleotide, dried down in tube format	339112
miRCURY LNA miRNA Custom Detection Probe (1 nmol)	1 nmol oligonucleotide, dried down in tube format	339115
miRCURY LNA miRNA Custom Detection Probe (10 nmol)	10 nmol oligonucleotide, dried down in tube format	339116
miRCURY LNA miRNA ISH Buffer Set (FFPE)	2x Formamide-free miRNA ISH buffer (25 ml), 1.25 ml Proteinase K Solution; for 1000 slides	339450
miRCURY LNA miRNA ISH Buffer and Control	Scramble miRNA Negative Control Probe (double DIG), U6 snRNA Positive Control Probe (5′ DIG), 2x Formamide-free miRNA ISH buffer (25 ml), 1.25 ml Proteinase K Solution; for 400 slides	339459
Related products		
miRCURY LNA miRNA ISH Optimization Kit (FFPE) 1	hsa-miR-1-3p LNA probe (double DIG), Scramble LNA Negative Control Probe (double DIG), U6 LNA Positive Control Probe (5′ DIG), 2x Hybridization Buffer, Proteinase K Solution; for 400 slides	339451

Product	Contents	Cat. no.
miRCURY LNA miRNA ISH Optimization Kit (FFPE) 2	hsa-miR-21-5p LNA probe (double DIG), Scramble LNA Negative Control Probe (double DIG), U6 LNA Positive Control Probe (5′ DIG), 2x Hybridization Buffer, Proteinase K Solution; for 400 slides	339452
miRCURY LNA miRNA ISH Optimization Kit (FFPE) 3	hsa-miR-122-5p LNA probe (double DIG), Scramble LNA Negative Control Probe (double DIG), U6 LNA Positive Control Probe (5′ DIG), 2x Hybridization Buffer, Proteinase K Solution; for 400 slides	339453
miRCURY LNA miRNA ISH Optimization Kit (FFPE) 4	hsa-miR-124-3p LNA probe (double DIG), Scramble LNA Negative Control Probe (double DIG), U6 LNA Positive Control Probe (5′ DIG), 2x Hybridization Buffer, Proteinase K Solution; for 400 slides	339454
miRCURY LNA miRNA ISH Optimization Kit (FFPE) 5	hsa-miR-126-3p LNA probe (double DIG), Scramble LNA Negative Control Probe (double DIG), U6 LNA Positive Control Probe (5′ DIG), 2x Hybridization Buffer, Proteinase K Solution; for 400 slides	339455
miRCURY LNA miRNA ISH Optimization Kit (FFPE) 7	hsa-miR-145-5p LNA probe (double DIG), Scramble LNA Negative Control Probe (double DIG), U6 LNA Positive Control Probe (5′ DIG), 2x Hybridization Buffer, Proteinase K Solution; for 400 slides	339457
miRCURY LNA miRNA ISH Optimization Kit (FFPE) 8	hsa-miR-205-5p LNA probe (double DIG), Scramble LNA Negative Control Probe (double DIG), U6 LNA Positive Control Probe (5′ DIG), 2x Hybridization Buffer, Proteinase K Solution; for 400 slides	339458

Product	Contents	Cat. no.
miRCURY LNA miRNA ISH Optimization Kit (FFPE) 9	hsa-miR-223-3p LNA probe (double DIG), Scramble LNA Negative Control Probe (double DIG), U6 LNA Positive Control Probe (5′ DIG), 2x Hybridization Buffer, Proteinase K Solution; for 400 slides	339459

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