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***In situ* hybridization detection of microRNAs**

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Abstract

microRNAs (miRNAs) are endogenous ~22 nucleotide RNAs that play critical roles in many cellular processes including cell differentiation, proliferation and apoptosis. The analysis of spatiotemporal expression of miRNAs is important for dissecting their roles in development and during physiological/pathophysiological processes. *In situ* hybridization is a powerful technology that allows cellular localization. miRNA *in situ* hybridization, however, has been challenging because of the low affinity of conventional RNA or DNA probes due to the small sizes of miRNAs. Here, we describe a protocol for miRNA *in situ* hybridization on mouse tissue cryosections using locked nucleic acid (LNA) probes. LNA probes demonstrate a much higher affinity to their complementary RNAs compared to conventional RNA and DNA oligo probes, which allows detection of miRNAs in tissue sections with excellent specificity.

Keywords

microRNAs; *in situ* hybridization; locked nucleic acid; cryosections

1. Introduction

As post-transcriptional regulators, miRNAs play important roles in the regulation of gene expression and thus are important in many biological processes [1,2]. Spatiotemporal expression of specific miRNAs in specific organs can provide important clues on their potential functions. Therefore, it is usually the first essential step to determine the expression profiles and cellular localization of specific miRNAs. Since all organs contain multiple cell types with different proportions, expression profiling assays, e.g. miRNA Northern blots or miRNA PCR, cannot reveal cellular sources or localizations of miRNAs expression. *In situ* hybridization represents an ideal method to reveal cellular localization of mRNAs and long non-coding RNAs [3]. miRNA *in situ* hybridization has been challenging because conventional RNA or DNA probes display low affinity to their targets due to the small sizes of miRNAs (~22nt) and thus low specificity. The development of locked nucleic acid (LNA) appears to have solved this problem. LNA is a class of bicyclic high-affinity RNA analogs in which the furanose ring of the ribose sugar is chemically locked in an RNA mimicking N-type (C3'-endo) conformation by the introduction of an O2', C4'- methylene bridge. Therefore, compared to traditional DNA probes, the LNA modified DNA probes display a much higher hybridization affinity toward the complementary RNA molecules [4,5]. Moreover, the increased thermal stability of the LNA-DNA/RNA duplex make it possible to generate probes as short as ~20 nt with high melting temperatures ($\geq 70^{\circ}\text{C}$), providing the basis for high stringency required by miRNA *in situ* hybridization [6,7]. Because of these properties, LNA modified DNA probes have become the first choice in miRNA *in situ*

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hybridization assays. Here we describe a step-by-step protocol for miRNA *in situ* hybridization using LNA probes. This protocol is designed for digoxigenin (DIG)-labeled LNA probes, which can be visualized using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) in order to enhance the signal strength. The NBT/BCIP visualization generates a blue precipitate that can be detected with light microscopy.

2. Materials

2.1 Labeling miRCURY LNA miRNA Detection Probes

1. miRCURY LNA miRNA detection probes (Exiqon)
2. DIG Oligonucleotide Tailing Kit, 2nd Generation (Roche)
3. 0.1M EDTA (pH8.0) (Fisher Scientific)

2.2 Purification of Probes Using MicroSpinG-25 Column

1. Illustra MicroSpin G-25 Columns (GE Healthcare)

2.3 Determination of Labeling Efficiency

1. Control DIG-dUTP/dATP tailed oligonucleotide (2.5 pmol/ μ l, provided in DIG Oligonucleotide Tailing Kit).
2. 1 $\times$ Phosphate buffered saline (PBS) buffer diluted from 10 $\times$ PBS (Fisher BioReagents)
3. Block solution [1% bovine serum albumin (BSA)] prepared from BSA powder (Fisher Scientific)
4. Antibody solution: Dilute 10 μ l of anti-DIG alkaline phosphatase conjugated antibody (Roche) in 10 ml of Block solution.
5. NBT/BCIP solution: Prepared from NBT/BCIP Stock Solution (Roche) (see the manufacturer's instruction).
6. TE Buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) prepared from Tris Base (Fisher Scientific) and EDTA (Fisher Scientific)

2.4 Preparation of Frozen Sections

1. 1 $\times$ PBS
2. Acetone (Fisher Scientific)
3. 5% Sucrose and 20% Sucrose: for 5% Sucrose, add 2.5g of Sucrose (Sigma) in 5 ml of 10 $\times$ PBS, and yield a final volume of 50 ml with nuclease-free water; for 20% Sucrose, add 10g of Sucrose in 5 ml 10 $\times$ PBS, and bring it to 50 ml with nuclease-free water. Then prepare 10% Sucrose, 12.5% Sucrose, 15% Sucrose from these two stocks 5% and 20% Sucrose solutions.
4. 20% Sucrose/OCT solution (4 ml): Mix 2 ml of 20% Sucrose and 2 ml of O.C.T. Compound (Tissue-Tek).
5. 2-Methylbutane (ACROS Organics).

2.5 In Situ Hybridization

1. 4% PFA: add 16g of paraformaldehyde (EMD Biosciences) in 40 ml of 10 $\times$ PBS, and yield a final volume of 400 ml with nuclease-free water, and adjust pH to 7.4-7.6.

* Use preheated water (60°C) to dissolve paraformaldehyde.

2. IsHyb In Situ Hybridization (ISH) Kit (Biochain). This kit contains Pre-hybridization Solution, Hybridization Solution, 20× SSC Buffer, 100× Blocking Solution, anti-DIG alkaline phosphatase conjugated antibody, 10× Alkaline Phosphatase Buffer, and NBT/BCIP.

2.6 Washing

1. 2× SSC, 1× SSC, and 0.2× SSC prepared from 20× SSC.
2. 1× Blocking solution prepared from 100× Blocking Solution provided in the ISH kit.

2.7 Immunological Detection

1. Anti-DIG alkaline phosphatase conjugated antibody solution (50 µl) containing 0.1 µl of the antibody and 50 µl of 1× Blocking Solution.
2. 1× Alkaline Phosphatase Buffer prepared from 10× Alkaline Phosphatase Buffer.
3. NBT/BCIP solution containing 6.6 µL of NBT and 3.3 µl of BCIP in 1 ml 1× Alkaline Phosphatase Buffer.
4. Aqua-Mount (Lerner Laboratory).

3. Methods

3.1. Labeling miRCURY LNA miRNA Detection Probes

1. miRCURY LNA miRNA detection probes (25 pmol/µl) can be purchased from Exiqon.
2. Set a reaction mixture on ice with a final volume of 20 µl in a PCR tube containing 4 µl (100 pmol) of a LNA miRNA detection probe, 4 µl of Reaction buffer, 4 µl of CoCl₂-solution, 1 µl of DIG-dUTP solution, 1 µl of dATP solution, and 1 µl of 400U Terminal transferase, and 5 µl of nuclease-free water.
3. Mix them well and centrifuge briefly.
4. Incubate the mixture at 37°C for 30 min and then place on ice.
5. Stop reaction by adding 5µl of 0.1M EDTA (pH 8.0).

3.2 Purification of DIG-labeled LNA Probes

1. For preparation of a column, resuspend the resin in the column by vortexing. Loosen the cap by a one-quarter turn and snap off the bottom closure. Place the column in the supplied collection tube. Pre-spin the column at 1000× g for 1 min.
2. Transfer the column to a clean 1.7 ml microcentrifuge tube. Slowly apply the DIG-labeled LNA probe sample to the center of the resin.

* Do not to disturb the resin bed.

3. Centrifuge at 1000× g for 4 min. The purified probes are collected at the bottom of the tube.
4. Measure purified probe concentrations using NanoDrop or another spectrophotometer.

* Probe concentration is ~30 ng/µl.

5. Aliquot labeled probes into multiple tubes and store at -20°C or -80°C .

3.3 Determination of Labeling Efficiency (Fig.1)

1. Dilute DIG-labeled LNA probes (30 ng/ μl) and the control DIG-dUTP/dATP tailed oligonucleotide (2.5 pmol/ μl) in nuclease-free water at serial dilutions 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} in a volume of 50 μl .
2. Spot 1 μl of undiluted probe and 1 μl of each dilution onto nitrocellulose.
3. Air dry membrane and UV-crosslink both sides to fix the nucleic acid
4. Equilibrate the membrane with $1\times$ PBS for 3 min and incubate it in 10 ml of $1\times$ PBS in a container containing 1% BSA at room temperature (RT) for 30 min.
5. Prepare 10 ml of Antibody solution freshly (see Materials), and incubate the membrane at RT for 30 min.
6. Wash the membrane with $1\times$ PBS twice (10 min each).
7. Add 10 ml of NBT/BCIP solution to the membrane and incubate for 10 min.
8. Stop the reaction by rinsing the membrane with TE buffer several times.

3.4 Preparation of Frozen Sections

1. Dissect a mouse testis tissue.
2. Wash tissue with $1\times$ PBS.
3. Fix tissue in 4 ml of acetone in a tube at 4°C for 30 min.
4. Wash with 5% Sucrose.
5. Incubate with 10% Sucrose, 12.5% Sucrose, and 15% Sucrose by gentle shaking at 4°C for 30 min, respectively.
6. Finally transfer the tissue to 20% Sucrose, shake at 4°C for overnight.
7. Put the tissue in 4 ml of 20% Sucrose/OCT solution, shake at 4°C for 30 min.
8. Freeze the tissue in the mixture in 2-Methylbutane cooled in liquid nitrogen and store the block at -80°C until used.
9. Cut sections of 10 μm on cryostat and mount onto slides.
10. Air dry sections at least 20 min, but for no longer than 3 hours (hrs)
11. Proceed to *In Situ* Hybridization or store them at -20°C (frost -free). (see **Note 1**)

3.5 In Situ Hybridization

1. Warm the frozen slides at RT. Dry them at 50°C for 15 min.
2. Fix the slides with 4% PFA at RT for 20 min.
3. Wash the slides three times (5 min each) with $1\times$ PBS at RT.
4. Pre-hybridize the slides with Pre-hybridization Solution at 60°C for 3 hrs.
* Pre-hybridization temperature should be 15°C below T_m of miRCURYTM LNA detection probes (e.g. 60°C calculated from 75°C of mmu-miR-883a-3p T_m). (see **Note 2**)
5. Prepare hybridization mixture by adding 0.2 μL of DIG-labeled LNA probe to 20 μl of hybridization solution.

6. Heat hybridization mixture at 65 °C for 5 min to linearize probes and then chill on ice.
7. Apply the prepared hybridization mixture onto the slides. Hybridize the slides at 55 °C for 12 hrs.
 - * Hybridization temperature should be 20°C below T_m of miRCURY™ LNA detection probes (e.g. 55°C calculated from 75°C of mmu-miR-883a-3p T_m). (see **Note 2**)

3.6 Washing

1. Wash the slides with 2× SSC at 55 °C for 10 min.
 - * Washing temperature should be 20°C below T_m of miRCURY™ LNA detection probes (e.g. 55°C calculated from 75°C of mmu-miR-883a-3p T_m). (see **Note 2**)
2. Wash the slides with 1× SSC at the same temperature 55 °C for 10 min.
3. Wash the slides with 0.2× SSC twice (20 min each) at 37°C.
4. Incubate the slides with 1× Blocking Solution at RT for 1 hr. (see **Note 3**)

3.7 Immunological Detection

1. Incubate the slides in 50 µl of the anti-DIG alkaline phosphatase conjugated antibody solution at 4°C for overnight.
2. Wash the slides with 1× PBS three times (10 min each) at RT.
3. Wash the slides with 1× Alkaline Phosphatase Buffer twice (5 min each) at RT.
4. Incubate the slides with NBT/BCIP solution in a dark room until a desired signal appears.
5. Discard the NBT/BCIP solution. Rinse the slides with distilled water.
6. Counterstain the slides with nuclear fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) or propidium iodide.
7. Mount the slides with Aqua-Mount.
8. Analyze on a microscope.

4. Note

1. Fresh sections are recommended for best results although several months old slides can still be usable.
2. It is important to determine optimal temperatures of pre-hybridization, hybridization and washing for each probe. Optimal temperatures can be calculated from the T_m of each probe, which is provided by the company (see ***In Situ Hybridization and Washing***).
3. Blocking step can be also performed at 4°C for overnight because longer incubation will generally give lower background.
4. We have used this protocol and examined localization of 3 X-linked miRNAs in the mouse testes [8].
5. To better demonstrate the cellular sources of miRNA *in situ* hybridization signals, conventional immunofluorescent staining of marker proteins can be performed after

the *in situ* hybridization procedures, as described in ref. ⁸. The fluorescent images from the red and green channels can then be merged with NBT/BCIP images (designated as the blue channel) using Adobe Photoshop software (Adobe).

6. Control experiments: Since both the 5' and the 3' strands of a stem-loop structure of a precursor miRNAs can be expressed in certain tissues [9], the traditional way of using the sense probe as a control probe is not applicable for miRNA *in situ* hybridization. We use either antisense LNA oligos with several mutations in their sequences, or DIG-labeled ("hot") LNA probes competed with 100X excessive amount of unlabeled ("cold") LNA probes during hybridization. The later method is more economic (no need to order mutant LNAs as controls), and convenient. Briefly, the concentrations of the DIG-labeled and unlabeled LNA probes are adjusted to 5pmol/μl and 25pmol/μl, respectively. Prepare the control hybridization mixture by adding 0.2 μL of DIG-labeled "hot" LNA probe and 4.0 μL of unlabeled "cold" LNA probe to 20 μl of hybridization solution. This will result in 100× excessive numbers of "cold" probe compared to "hot" probe in the hybridization solution. Proceed to hybridization and the rest of the steps as described in 3.5-3.7.

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References

1. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004; 116:281–97. [PubMed: 14744438]
2. Kim VN, Nam JW. Genomics of microRNA. *Trends Genet*. 2006; 22:165–73. [PubMed: 16446010]
3. Mitchell BS, Dhami D, Schumacher U. *In situ* hybridisation: a review of methodologies and applications in the biomedical sciences. *Med Lab Sci*. 1992; 49:107–18. [PubMed: 1283195]
4. Kloosterman WP, Wienholds E, de Bruijn E, Kauppinen S, Plasterk RH. *In situ* detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. *Nat Methods*. 2006; 3:27–9. [PubMed: 16369549]
5. Wheeler G, Valoczi A, Havelda Z, Dalmay T. *In situ* detection of animal and plant microRNAs. *DNA Cell Biol*. 2007; 26:251–5. [PubMed: 17465891]
6. Obernosterer G, Martinez J, Alenius M. Locked nucleic acid-based *in situ* detection of microRNAs in mouse tissue sections. *Nat Protoc*. 2007; 2:1508–14. [PubMed: 17571058]
7. Silahtaroglu AN, Nolting D, Dyrskjot L, Berezhikov E, Moller M, Tommerup N, Kauppinen S. Detection of microRNAs in frozen tissue sections by fluorescence *in situ* hybridization using locked nucleic acid probes and tyramide signal amplification. *Nat Protoc*. 2007; 2:2520–8. [PubMed: 17947994]
8. Song R, Ro S, Michaels JD, Park C, McCarrey JR, Yan W. Many X-linked microRNAs escape meiotic sex chromosome inactivation. *Nat Genet*. 2009; 41:488–93. [PubMed: 19305411]
9. Ro S, Park C, Young D, Sanders KM, Yan W. Tissue-dependent paired expression of miRNAs. *Nucleic Acids Res*. 2007; 35:5944–53. [PubMed: 17726050]

	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}
mir-718-3p +
mir-718-3p -
mir-883a-3p +
mir-883a-3p -
Positive Control

Fig.1.

Labeling efficiency test. '+' stands for DIG-labeled probe, and '-' for unlabeled probe. DIG-dUTP/dATP tailed oligonucleotide provided in Roche DIG Oligonucleotide Tailing Kit was used as a positive control. 10^0 , undiluted probes. 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} , serial diluted probes.

Fig.2.

In situ hybridization analyses of mir-92a-3p in adult mouse testes. (A) mir-92a-3p is detected in round spermatids (rSd) at lower levels and in elongated spermatids (eSd) at higher levels, whereas early spermatogenic cell types including spermatogonia and spermatocytes (Sp) show no detectable hybridization signals. (B) DAPI counterstaining of cell nuclei in the section shown in panel A. (C) Control *in situ* hybridization using DIG-labeled mir-92a-3p probe competed with 100× excessive amounts of unlabeled mir-92a-3p probe. (D) DAPI counterstaining of cell nuclei in the section shown in C. Magnification: 400X.