

Chapter 2

MicroRNA In Situ Hybridization on Whole-Mount Preimplantation Embryos

Karen Goossens, Luc Peelman, and Ann Van Soom

Abstract

Whole-mount in situ hybridization (WISH) using antisense probes is widely used to visualize RNA sequences in embryos and to determine the precise site of expression in the different cells or tissues. The target sequence is hybridized with an antisense RNA probe, followed by visual or fluorescence detection to measure the site and level of expression. However, the detection of short RNA molecules is hampered by the reduced stringency of the probes for short transcripts. Here, we describe a procedure for WISH detection of short RNA molecules, like miRNAs, in mammalian preimplantation embryos using LNA-modified probes with high sensitivity and specificity.

Key words MicroRNA, Blastocyst, Embryo, In situ hybridization, LNA

1 Introduction

MicroRNAs (miRNAs) are endogenous, single-stranded noncoding RNAs of around 22 nucleotides. Recent studies have revealed that miRNAs play essential roles in gene regulation during early embryo development and the biogenesis of stem cells [1–5]. To identify the function of specific miRNAs during the very beginning of development from a single cell to an organism composed of different cell types and tissues, it is important not only to analyze their expression in the entire organism but also to determine the precise site of expression in the different cells or tissues. The isolation of pure cell populations out of heterogeneous tissues or organisms is not always feasible. As an alternative for the separation of cells and tissues, whole-mount in situ hybridization (WISH) can be used to visualize the site and level of expression of specific miRNAs in the whole specimen [6, 7]. The procedure consists of hybridization with a specific probe, followed by colorimetric or fluorescent detection.

WISH on embryo samples is frequently used for mRNA detection [8, 9]. However, due to the short length of miRNAs and their

close association with large protein complexes, the use of in situ hybridization for miRNA detection is more challenging. Thanks to the development and improvement of new types of probes, in situ hybridization can now be successfully used for the detection of very short RNA molecules, like miRNAs, with a high sensitivity and specificity. The most popular type of new probes is the Locked Nucleic Acid or LNA probe [10–12]. LNAs are modified nucleotides that contain a methylene bridge between the 2'-O and the 4'-C on the ribose ring that locks the structure into a high-binding-affinity, RNA-mimicking conformation [10, 13]. LNA molecules are able to form base pairs with DNA and RNA molecules according to standard Watson-Crick base pairing rules. Each incorporated LNA nucleotide increases the melting temperature of the LNA-DNA/RNA duplex by 2–10 °C, resulting in an enhanced stringency for probes as short as ~20 nt and enabling to discriminate between single nucleotide differences and hence between closely related miRNA family members. Other examples of probes that can be used for miRNA in situ hybridization are the morpholinos [7] or 2'-O-methyl RNA probes (2OMe) [14].

In this chapter, a detailed protocol for WISH on bovine blastocysts using DIG-labeled LNA probes in combination with colorimetric detection is described.

2 Materials

One of the major challenges when working with RNA is the prevention of RNA degradation. RNA is extremely susceptible to degradation and easily degraded by RNase enzymes that can be endogenously present in the sample but that can also be introduced during the WISH procedure. Consequently, all recipients, products, and solutions used for WISH should be RNase-free (*see Note 1*).

2.1 Equipment

1. Gloves.
2. RNase inactivating solution (e.g., RNase AWAY).
3. 10 ml screw top tubes.
4. 50 ml screw top tubes.
5. 1.5 ml eppendorf tubes.
6. Petri dishes (Ø 35 mm).
7. Multi-well plates (4-well up to 24-well plates can be used).
8. Embryo manipulation system (e.g., Unopette system, mouth pipetting system).
9. Microcentrifuge.
10. Vortex.

11. Hybridization oven.
12. Hybridization box.
13. Horizontal shaker (cooled 4 °C).
14. Stereomicroscope.
15. Bright-field microscope.
16. Optional: fluorescent microscope or confocal microscope (*see Note 2*).

2.2 Fixation

1. Wash buffer: RNase-free phosphate-buffered saline (PBS). Add 0.2 g KH_2PO_4 , 0.2 g KCl, 8 g NaCl, and 2.2 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ to 800 ml distilled, DEPC-treated water; adjust to pH 7.4 with 100 mM NaOH; and adjust the volume to 1 L (*see Note 3*).
2. Fixative: 4 % paraformaldehyde (PFA). Dissolve 40 g of paraformaldehyde in 1 L of preheated (60 °C) distilled, DEPC-treated water. Adjust to pH 7.4 with 100 mM NaOH. Filter the solution using a 0.45- μm membrane filter. Store the stock solution at -20 °C in aliquots.
3. 100 % methanol.

2.3 Rehydration and Protein Digestion (See Note 5)

1. 0.1 % PBST (PBS with Tween-20): dissolve 500 μl Tween-20 in 500 ml RNase-free PBS (*see Note 4*).
2. Series of methanol (MeOH): 25 % MeOH; 50 % MeOH; 75 % MeOH diluted in 0.1 % PBST.
3. Proteinase K: 20 mg/ml stock diluted in 0.1 % PBST to 10 $\mu\text{g}/\text{ml}$.

2.4 Pre-hybridization

1. Sheep serum.
2. BSA fraction V protease-free.
3. Blocking buffer: 0.1 % PBST-2 % sheep serum-BSA (for 5 ml). Dissolve 10 mg BSA in 4.9 ml PBST, and add 100 μl of sheep serum.
4. Anti-digoxigenin-AP, Fab (Roche): make a 1:1,000 dilution of the antibody in PBST-2 % sheep serum-BSA blocking buffer.

2.5 Hybridization

1. Hybridization buffer (HYB-) (for 50 ml): 30 ml deionized formamide (*see Note 6*), 12 ml 20 \times Saline Sodium Citrate (SSC), 50 μl Tween-20, 460 μl 1 M citric acid, 7.49 μl DEPC-treated water.
2. Hybridization buffer+heparin+tRNA (HYB+) (for 50 ml): add 2.5 mg of heparin (final concentration 50 $\mu\text{g}/\text{ml}$), and add 25 mg of yeast tRNA (final concentration 500 $\mu\text{g}/\text{ml}$), per 50 ml of HYB-.

3. 5' and/or 3'DIG-labeled miRCURY LNA probe for specific miRNA of interest (Exiqon) (*see* **Note 7**).
4. 5' and/or 3'DIG-labeled miRCURY LNA scrambled control probe (Exiqon) (*see* **Note 8**).
5. 2× SSC: make a 1:10 dilution of 20× SSC in PBST.
6. 0.2× SSC: make a 1:100 dilution of 20× SSC in PBST.
7. Series of hybridization buffer (HYB– series) diluted in 2× SSC: 75 % HYB–/25 % 2× SSC; 50 % HYB–/50 % 2× SSC; 25 % HYB–/75 % 2× SSC.
8. Series of 0.2× SSC diluted in PBST: 75 % 0.2× SSC/75 % PBST; 50 % 0.2× SSC/50 % PBST; 25 % 0.2× SSC/75 % PBST.
9. Pre-absorbed anti-digoxigenin-AP: make a 1:5,000 dilution of the pre-absorbed anti-digoxigenin-AP in blocking buffer (*see* **Note 9**).

2.6 Staining

1. Staining buffer (for 50 ml): 5 ml of 1 M Tris–HCl pH 9.5, 2.5 ml of 1 M MgCl₂, 50 µl Tween-20. Add 42.45 ml DEPC-treated water. Filter the solution using a 0.45 µm syringe filter.
2. Nitrotertrazolium blue chloride (NBT; Sigma-Aldrich): dissolve 50 mg NBT in 700 µl dimethylformamide anhydride and 300 µl DEPC-treated water.
3. 5-Bromo-4-chloro-3-indolyl phosphate *p*-toluidine (BCIP; Sigma-Aldrich): dissolve 50 mg BCIP in 1 ml dimethylformamide anhydride.
4. Staining solution (for 50 ml): 225 µl of 50 mg/ml NBT, 175 µl of 50 mg/ml BCIP, 50 ml staining buffer (*see* **Note 10**).
5. Stop solution 1 mM EDTA in PBS (for 50 ml): 50 ml PBS, 100 µl 0.5 M EDTA.

2.7 Mounting

1. Methyl salicylate.
2. Siliconized microscopy slides (*see* **Note 11**).
3. Vaseline in plastic 10 ml syringe (*see* **Note 12**).
4. Glycerol with 25 mg/ml 1,4-diazabicyclo[2.2.2]octane mounting medium (DABCO) (*see* **Note 13**).

3 Methods

In order to prevent RNA degradation in the sample, apply RNase inactivating solution to all surfaces and equipment, wipe dry, and rinse with RNase-free water to remove the RNase inactivating solution. In addition, working with gloves during the entire procedure will further prevent contamination with RNases.

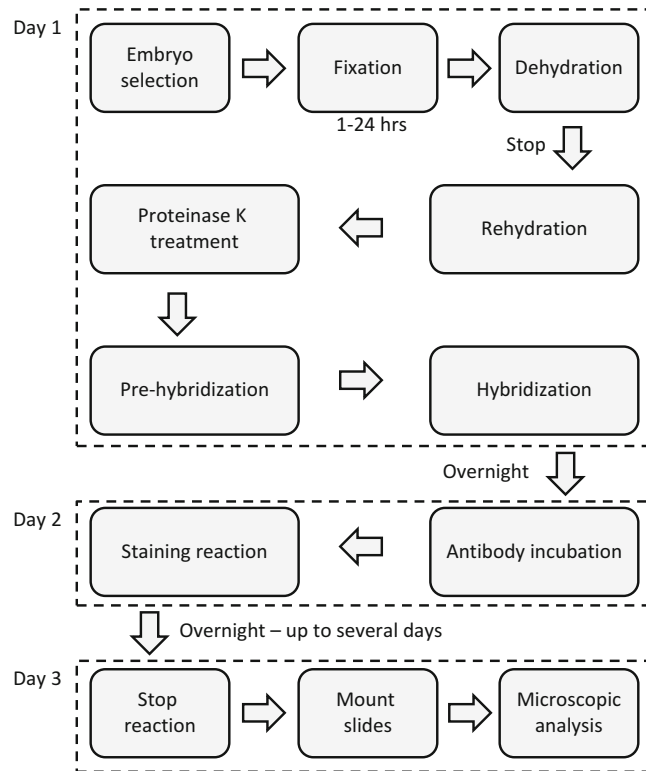


Fig. 1 Whole-mount in situ hybridization flowchart. The procedure takes a minimum of 3 days, as indicated by the *dotted boxes*

Transfer the embryos from one solution to the other under visual control, using a stereomicroscope and an embryo manipulation system.

When working with toxic solutions (e.g., PFA, methanol, formamide, NBT), work in a fume hood and take appropriate safety measurements.

The entire procedure will take a minimum of 3 days and is schematically presented in Fig. 1.

3.1 Sample Collection and Fixation

1. Bovine embryos are produced by routine in vitro methods as described by Vandaele et al. [15].
2. Blastocysts with good morphological characteristics are selected from the culture medium on day 8 post insemination (*see Note 14*).
3. Wash the blastocysts three times in PBS and put them immediately in a small petri dish (\varnothing 35 mm), filled with 4 % PFA.
4. Fix the blastocysts for 1–24 h at 4 °C (*see Note 15*).
5. After fixation, dehydrate the embryos in ice-cold 100 % methanol at –20 °C for at least 2 h (*see Note 16*). Methanol can be used for long-term storage of fixed embryos (*see Note 17*).

3.2 Rehydration and Protein Digestion

1. Rehydrate the embryos by performing 5 min washes in ice-cold methanol series: 75 % methanol/25 % PBST, 50 % methanol/50 % PBST, and 25 % methanol/75 % PBST followed by four rinses in PBST. Small petri dishes or well plates can be used for washing steps. It is important to work on ice and to work with cooled solution to prevent RNA degradation (*see Note 18*).
2. Transfer the embryos to Proteinase K solution for 30 s at room temperature (*see Note 5*) and rinse them immediately for 5 min in cooled PBST.
3. Refix the embryos in 4 % PFA for 20 min at 4 °C.
4. After fixation, wash the embryos five times for 5 min in cooled PBST.

3.3 Pre-hybridization

1. Pre-warm the hybridization buffer with heparin and tRNA (HYB+) at the specific probe temperature (approximately theoretical T_m probe -20 °C; *see Note 19*).
2. Pre-hybridize the blastocysts for a minimum of 2 and a maximum of 5 h in 800 μ l pre-warmed HYB+ at the specific probe temperature in an hybridization oven (*see Notes 20 and 21*).
3. Meanwhile, dissolve 1 μ l of DIG-labeled miRCURY LNA probe per ml of HYB+ (final concentration 10 nM) (*see Note 22*). Do the same for the scrambled negative control probe.

3.4 Hybridization

1. Remove the embryos from the pre-hybridization buffer and divide them into three groups: a test group that will be incubated with a specific probe for the miRNA of interest, a negative control group that will be incubated with a scrambled negative control probe, and a double negative control group that stays overnight in HYB+.
2. Incubate the test group and the negative control group overnight in 10 nM DIG-labeled miRCURY LNA probe solution at the specific probe temperature (approximately theoretical T_m probe -20 °C; *see Note 20*) in a hybridization oven (*see Note 21*).
3. Pre-warm the hybridization buffer without heparin and tRNA (HYB-), the hybridization buffer series, and 2 \times and 0.2 \times SSC solution at the specific probe temperature.
4. The next morning, wash the embryos (all three groups) for 10 s in pre-warmed HYB- followed by 15 min washes in the hybridization buffer series: 75 % HYB-/25 % 2 \times SSC, 50 % HYB-/50 % 2 \times SSC, 25 % HYB-/75 % 2 \times SSC, and 2 \times SSC at the specific probe temperature.
5. Subsequently, wash the embryos in the 0.2 \times SSC series for 10 min at room temperature.

6. Finally, wash the embryos for 10 min in PBST at room temperature. Incubate the embryos for a minimum of 2 h in blocking buffer and put the plates on a horizontal shaker at room temperature.
7. Meanwhile, make a 1:1,000 dilution of the anti-digoxigenin-AP antibody in blocking buffer. Pre-absorb the antibody by adding bad-quality blastocysts or embryos from earlier developmental stages (*see Note 9*). Use approximately 5 embryos per 100 μ l of antibody solution. Incubate the antibody with the embryos for minimum 1 h at room temperature while shaking. Subsequently, centrifuge the pre-absorbed antibody solution for 5 min at $15,000\times g$. Take the supernatant consisting of pre-absorbed anti-digoxigenin-AP antibodies and dilute it $5\times$ in blocking solution to obtain a 1:5,000 final dilution.
8. Incubate the embryos of all three groups overnight in pre-absorbed and diluted anti-digoxigenin-AP antibody while continuously shaking on a horizontal shaker at 4 °C.

3.5 Staining

1. The next day, remove the anti-DIG antibody and wash the embryos five times for 20 min in cooled PBST at 4 °C, followed by three washes in staining buffer at room temperature for 20 min.
2. Replace the staining buffer by staining solution containing the nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyphosphate *p*-toluidine (NBT/BCIP) alkaline phosphatase substrate and incubate overnight at room temperature (*see Note 23*). During the staining reaction, keep the plates away from light in a hybridization box (*see Note 24*).
3. Stop the color reaction by adding stop solution. Embryos can be stored in stop solution until further evaluation.

3.6 Mounting

1. Dehydrate the embryos in 100 % methanol.
2. Wash them in methyl salicylate.
3. Mount 2–3 embryos per droplet of DABCO (*see Note 13*) on siliconized microscopy slides (*see Note 11*) with vaseline bridges (*see Note 12*).
4. Analyze the samples with a bright-field microscope (*see Note 2*). The results of miR-155 expression analysis in bovine blastocysts by WISH are shown in Fig. 2 [6].

4 Notes

1. RNase-free water can either be purchased or self-made by running ultrapure water through a nuclease filter which retains RNases or by diethyl pyrocarbonate (DEPC) treatment of

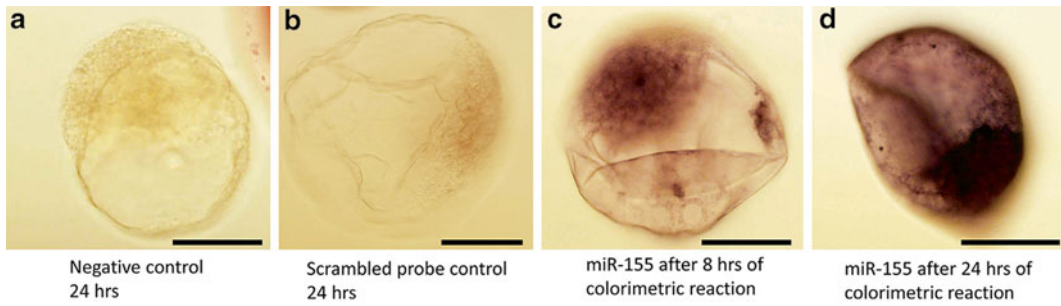


Fig. 2 Whole-mount in situ hybridization for bta-miR-155 on bovine blastocysts. **(a):** Negative control staining by omitting the LNA probe. **(b):** Negative control using the scrambled negative control LNA probe. **(c):** WISH for bta-miR-155 after 8 h of colorimetric reaction in staining solution. **(d):** WISH for bta-miR-155 after 24 h of colorimetric reaction in staining solution. Expression of bta-miR-155 was mainly detected in the ICM. A weaker expression was observed in the TE. Based on Goossens et al. (2012) [6]. Scale bar: 50 μ M

distilled water. Glass slides, devices, and recipients can be dry heat sterilized in an oven at 180 °C for a minimum of 2 h. All recipients and devices that cannot be sterilized can be wiped with an RNase inactivating solution. Plastics should be bought RNase/DNAse and protease-free when possible.

2. A fluorescent microscope is required when working with fluorescent labeled probes or Fast Red detection [4, 16, 17]. In addition, confocal imaging can be used for more exact miRNA localization in fluorescent stained whole-mount samples.
3. Instead of PBS, another physiological salt solution can also be used.
4. Pipette Tween-20 slowly to avoid air bubble formation; vortex well to dissolve Tween-20 in PBS.
5. Proteinase K (PK) digestion breaks RNA/protein complexes and facilitates infiltration of the probes. Protein digestion with PK should be optimized for each embryonic stage. Up to the blastocyst stage, PK treatment is basically not necessary. However, we had better results with regard to the signal to noise ratio when applying a sort PK treatment on the blastocyst samples. For later developmental stages, longer incubation times are required. For the optimal incubation times of mouse embryos and embryoid bodies, we refer to Piette et al. [8]. For embryos of other species, incubation times should be experimentally determined.
6. The formamide concentration should be empirically determined for each probe and can vary between 50 % and 65 %, depending on the hybridization strength of the probe.
7. In this protocol, the use of DIG-labeled LNA probes in combination with colorimetric NBT/BCIP detection is described (Fig. 3). Probes are available with a selection of 3' and 5' labels

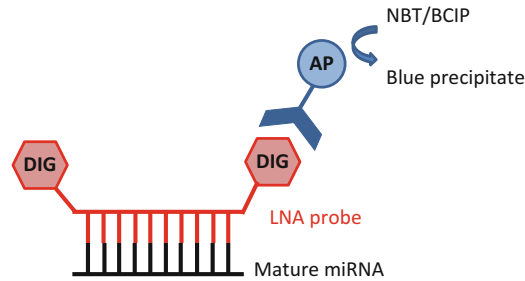


Fig. 3 Principle of colorimetric miRNA detection with LNA probes. The DIG-labeled LNA™ probe binds specifically to the mature miRNA of interest. Alkaline phosphatase (AP)-conjugated anti-DIG antibodies bind to the DIG label. AP converts the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate resulting in a blue precipitation that can easily be visualized using light microscopy

(DIG, biotin, fluorescein, amino; Exiqon). For low abundant miRNAs, it is recommended to use probes that are labeled at both 3' and 5' end. Probes can also purchased unlabeled and labeled in the laboratory using digoxigenin-11-ddUTP and terminal transferase (DIG Oligonucleotide 3'-End 2nd Generation Labeling Kit, Roche).

Dilute LNA probes in RNase-free water to a stock concentration of 10 μ M, aliquot them, and store them at -80°C . Optimal working concentrations range between 10 and 40 nM but should be experimentally determined for each sample type.

8. It is very important to include negative controls, by using scrambled negative control probes and by omitting the probe, in order to test for the specificity of the probe and the color reaction. Scrambled negative control probes are commercially available.
9. Antibody pre-absorption is required to reduce background signals.
10. DIG-labeled antibodies can be used with two different alkaline phosphatase color reactions, NBT/BCIP and Fast Red. Fast Red is described to be less sensitive and to fade very quickly, but it is fluorescent, what makes it suitable for fluorescent and confocal imaging [16, 17].
11. Siliconized microscopy slides are preferentially used to avoid spreading of the droplet of mounting medium. Siliconized microscopy slides can be commercially obtained or made in the laboratory using silicone solution (Serva).
12. Vaseline bridges are required to prevent crushing of the embryos. Put a small droplet of vaseline at the four corners of the microscopy slide before attaching the cover glass. As an

alternative spacers or special microscopy slides with single or multiple depressions can be used.

13. Instead of DABCO, another mounting medium like Permount or VectaShield can also be used.
14. In this protocol bovine embryos at the blastocyst stage are being used. The procedure can easily be adapted to embryos of other developmental stages or other species.
15. Fixation times from 1 up to 24 h have been tested and did not result in any difference in outcome.
16. During methanol incubation, the blastocysts can stick to the bottom of the petri dish or well plate. Use a small needle or pipette tip to carefully release them from the bottom while gently moving the plate. The methanol will make the embryos fragile so take care not to break them.
17. When methanol is used for long-term storage of the samples, the petri dish has to be sealed carefully to prevent evaporation of the methanol.
18. Instead of working on an ice bath, samples can be incubated in the fridge at 4 °C.
19. The optimal hybridization temperature is around the theoretical melting temperature (T_m) -20 °C. However, the exact hybridization temperature should be tested for each probe. We recommend performing a hybridization temperature course of 7 different temperatures for the miRNA probe of interest, respectively, at 2 °C, 4 °C, and 6 °C below and above the theoretical optimal temperature.
20. Embryos will become translucent during incubation in hybridization buffer. This makes them difficult to see. Moreover, they sometimes float on top of the solution. Focus on top of the liquid and gently try to push the embryos to the bottom of the plate, using a small needle or pipette tip.
21. A water bath can be used instead of an hybridization oven; however, there is a higher risk of contamination when using a water bath.
22. The amount of necessary probe solution is determined by the size of the well plates. Samples must be completely covered with probe solution.
23. Longer incubation times, up to several days, might be necessary to detect lowly expressed miRNAs. Monitor the staining intensity using a stereomicroscope.
24. During the staining reaction, protect the samples from light. Place the well plates in a metal box (hybridization box) or wrap them in aluminum foil. Light protection is especially important when working with fluorescent probes. For longer incubation times, put wet tissue paper in the box, to prevent evaporation.

References

1. Goossens K, Mestdagh P, Lefever S et al (2013) Regulatory microRNA network identification in bovine blastocyst development. *Stem Cells Dev* 22:1907–1920
2. Wang P, Cui J, Zhao C et al (2013) Differential expression of microRNAs in 2-cell and 4-cell mouse embryos. *Zygote*:1–7
3. Rosenbluth EM, Shelton DN, Sparks AE et al (2013) MicroRNA expression in the human blastocyst. *Fertil Steril* 99:855.e3–861.e3
4. Hossain MM, Salilew-Wondim D, Schellander K et al (2012) The role of microRNAs in mammalian oocytes and embryos. *Anim Reprod Sci* 134:36–44
5. Sharma A, Wu JC (2013) MicroRNA expression profiling of human-induced pluripotent and embryonic stem cells. *Methods Mol Biol* 936:247–256
6. Goossens K, De Spiegelaere W, Stevens M et al (2012) Differential microRNA expression analysis in blastocysts by whole mount in situ hybridization and reverse transcription quantitative polymerase chain reaction on laser capture microdissection samples. *Anal Biochem* 423:93–101
7. Lagendijk AK, Moulton JD, Bakkers J (2012) Revealing details: whole mount microRNA in situ hybridization protocol for zebrafish embryos and adult tissues. *Biol Open* 1:566–569
8. Piette D, Hendrickx M, Willems E et al (2008) An optimized procedure for whole-mount in situ hybridization on mouse embryos and embryoid bodies. *Nat Protoc* 3:1194–1201
9. Darnell DK, Stanislaw S, Kaur S et al (2010) Whole mount in situ hybridization detection of mRNAs using short LNA containing DNA oligonucleotide probes. *RNA* 16:632–637
10. Petersen M, Nielsen CB, Nielsen KE et al (2000) The conformations of locked nucleic acids (LNA). *J Mol Recognit* 13:44–53
11. Kloosterman WP, Wienholds E, de Bruijn E et al (2006) In situ detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. *Nat Methods* 3:27–29
12. Thomsen R, Nielsen PS, Jensen TH (2005) Dramatically improved RNA in situ hybridization signals using LNA-modified probes. *RNA* 11:1745–1748
13. Wengel J, Vester B, Lundberg LB et al (2003) LNA and alpha-L-LNA: towards therapeutic applications. *Nucleosides Nucleotides Nucleic Acids* 22:601–604
14. Soe MJ, Moller T, Dufva M et al (2011) A sensitive alternative for MicroRNA in situ hybridizations using probes of 2'-O-methyl RNA plus LNA. *J Histochem Cytochem* 59:661–672
15. Vandaele L, Mateusen B, Maes D et al (2006) Is apoptosis in bovine in vitro produced embryos related to early developmental kinetics and in vivo bull fertility? *Theriogenology* 65:1691–1703
16. Obernosterer G, Martinez J, Alenius M (2007) Locked nucleic acid-based in situ detection of microRNAs in mouse tissue sections. *Nat Protoc* 2:1508–1514
17. Hossain MM, Ghanem N, Hoelker M et al (2009) Identification and characterization of miRNAs expressed in the bovine ovary. *BMC Genomics* 10:443

In Situ Hybridization Protocols

Nielsen, B. (Ed.)

2014, XI, 275 p. 56 illus., 52 illus. in color., Hardcover

ISBN: 978-1-4939-1458-6

A product of Humana Press