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LNA-based *in situ* hybridization detection of mRNAs in embryos

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Summary

In situ hybridization (ISH) in embryos allows the visualization of specific RNAs as a readout of gene expression during normal development or after experimental manipulations. ISH using short DNA probes containing Locked Nucleic Acid nucleotides (LNAs) holds the additional advantage of allowing the detection of specific RNA splice variants, or of closely related family members that differ in only short regions, creating new diagnostic and detection opportunities. Here we describe methods for using short (14–24 nt) DNA probes containing LNA nucleotides to detect moderately to highly expressed RNAs in whole chick embryos during the first five days of embryonic development. The protocol is easily adaptable for use with embryos of other vertebrate species.

Keywords

chicken embryo; *Gallus gallus*; *in situ* Hybridization; Locked Nucleic Acids; LNA

1. Introduction

Basic and applied research in the areas of molecular, cellular, and developmental biology, evolution, agriculture and medicine are dependent on information about the biological output of the genome. The spatial and temporal extent of gene expression can be determined through hybridization of a probe that is antisense to the target RNA, followed by enzymatic or fluorescence visualization of the bound probe. When performed on tissues or whole embryos, the technique is called *in situ* hybridization (ISH). Most commonly, the technique is performed using an *in vitro* transcribed RNA that is antisense to the target RNA, with some nucleotides linked to an antigen such as Digoxigenin (DIG). The technique has typically been performed on sectioned tissues or on whole embryos using antisense RNA probes from 200–1000nt long. Probes of this length provide sufficient duplex stability for strong hybridization and dozens of incorporated DIG moieties to ensure high immunodetection sensitivity. When use of a much shorter probe is desired because the RNA in question is very short (e.g., microRNA, 22nt) or differs from related RNAs only in a short region (as in closely related genes or alternatively spliced RNA variants), the probe must be specifically engineered for stability and detection. Locked Nucleic Acids (LNAs) are an RNA nucleotide analogue that exhibits superior specificity, hybridization kinetics, and biostability [1–6]. LNAs contain a methylene bridge between the 2' O and the 4' C on the

ribose ring that ‘locks’ the ring into a high binding-affinity, RNA-mimicking conformation. Extensive testing has shown that short (14–25 nt) DNA oligonucleotides containing an LNA at every third position exhibit superior hybridization stability and melting temperatures that are compatible with ISH protocols [4]. Addition of DIG molecules internally and/or at either end of the LNA-containing oligonucleotide enables sensitive detection in fixed embryos and on tissue sections [7]. LNA probes for ISH detection can be designed in silico and ordered from commercial suppliers.

Here we provide a protocol for using LNA-containing DNA probes (hereafter called LNAs) for ISH detection of mRNAs in chicken embryos. This protocol has been optimized for probe concentration, hybridization time, number of DIG labels, LNA length, probe sequence and annealing location, target abundance, and processing temperatures and conditions [7]. This technique is easily adaptable for use with embryos from other species (Figure 1, Note 1).

2. Materials

2.1 Solutions and Reagents

Diethylpyrocarbonate (DEPC) Treated Water—add 0.1% DEPC to milliQ water, mix, let it sit at room temperature over night, then autoclave. Solutions for use after hybridization can be made with milliQ water unless otherwise noted.

Anti-DIG antibody (anti-DIG-AP Fab fragment, available from Roche)

CHAPS—3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate

Chicken Embryo Powder—Homogenize day 3–5 chick embryos in a minimum volume of PBS. Add 4 volumes of ice-cold acetone, mix and place on ice for 30min. Spin at 10,000x g for 10 min. Remove supernatant, wash pellet with ice-cold acetone and re-spin. Remove supernatant and spread pellet out to air dry in a clean mortar. Grind dry pellet to a fine powder and store in an airtight tube at 4 °C.

Chick Saline—123mM NaCl

KTBTw—50mM Tris-HCl, pH 7.5, 150mM NaCl, 10mM KCl, 1% Tween-20

LNAs—LNA probes can be ordered from several oligonucleotide suppliers, including Exiqon (www.exiqon.com). LNAs are available with a DIG label at the 5′ and 3′ ends, or only at one end. If several LNAs will be purchased, money can be saved by ordering LNAs that are DIG labeled only at the 5′ end, and then adding the 3′ DIG using the Roche DIG Oligonucleotide 3′-End 2nd Generation Labeling Kit.

¹This protocol is readily adaptable for use with other vertebrate species. Figure 1 shows the localization of the cardiac troponin T mRNA in chicken and *Xenopus* embryos.

LNA Working Stock and Final Solution—Working stock is 5 μ M in prehyb buffer. Dilute this stock solution 1:1000 in prehyb buffer to prepare final hybridization solution at 5nM.

Graded Methanol Series—25% in PBTw, 50% in PBS, 75% in water, 100%. Dehydration runs from low to high MeOH, Rehydration from high MeOH to low.

NBT/BCIP—For each ml of NTMT, add 3 μ l of 75mg/ml NBT (nitro-blue tetrazolium chloride) in DMF (dimethylformamide) and 2.3 μ l of 50mg/ml BCIP (5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt) in DMF/ml of NTMT. Concentrated stock aliquots of NBT and BCIP can be stored at -20°C , and then added to NTMT to make a working color reaction solution.

24 mm Netwell Inserts

NTMT—100mM Tris-HCl, pH 9.5, 100mM NaCl, 50mM MgCl₂, 0.1% Tween-20.

Paraformaldehyde (PFA) 4%—In a 2 liter beaker, warm 750 mL of DEPC treated water to approximately 60 $^{\circ}\text{C}$ (beaker should be warm but not hot to the touch). In a fume hood, add 40g of PFA prills and 2 NaOH pellets. Stir until PFA goes into solution, add 100 mL of 10x PBS and then HCl dropwise until pH is 7.2. Bring to 1 liter with DEPC treated water. Transfer to 50 mL conical tubes. Store in 4 $^{\circ}\text{C}$, use within 72 hours.

4% Paraformaldehyde/0.1% Glutaraldehyde in PBS

Phosphate Buffered Saline (PBS)—137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate (Na₂PO₄), 1.8 mM potassium dihydrogen phosphate (KH₂PO₄), pH 7.4

PBS plus 0.1% sodium azide

PBTw—PBS containing 0.1% Tween-20.

Prehybridization solution: (Prehyb)—50% deionized formamide, 5X saline sodium citrate (SSC; 0.75M sodium chloride, 75mM trisodium citrate, pH 7.0), 2% blocking powder (Roche), 0.1% Tween-20, 0.1% CHAPS, 50mg/ml yeast RNA, 5mM EDTA, 50mg/ml heparin. Adapted from Nieto et al [8], with Triton X-100 replaced with Tween-20.

Proteinase K—Prepare 150 μ l aliquots of 10mg/ml proteinase K in DEPC treated water. Store at -20°C until just before use. Individual lots of proteinase K vary in effective concentration. New lots should be tested over a concentration range to identify the optimal conditions for proteinase K treatment.

SSC Washing Buffers—First washes (3): 2xSSC with 0.1% CHAPS 0.1% Tween-20 and 0.1% Tween-20. Second washes (3): 0.2xSSC with 0.1% CHAPS 0.1% Triton X-100 and 0.1% Tween-20.

3. Methods

3.1 LNA Probe Design and Ordering

LNA probes can be designed using any standard probe design program. We design 20–24nt probes using Primer3 [9], though probes as short as 14nt may give good signal [7]. Probes are checked for self-complementarity and secondary structure using Exiqon's LNA Oligo Optimizer tool (www.exiqon.com). Screen each probe sequence against all known chicken sequences in NCBI using BLAST to ensure it is unique to the target RNA. LNA probes typically show reduced hybridization or fail to hybridize to targets with even single- or double-nucleotide mismatches, respectively [10,7]. Through careful optimization of probe concentration and hybridization parameters, abundant and moderately abundant mRNAs coding for structural, signaling, and transcriptional regulatory proteins can be detected. Finally, detection sensitivity can be increased by designing more than one LNA probe per target mRNA [7]. When using multiple probes, the final LNA concentration for all probes combined should be 5nM (see Note 2).

3.2 Embryo Collection and Preparation

- 1 Incubate fertile chicken eggs in a forced-draft, humidified incubator at 37.5°C for 1–5 d, depending on the embryo stages desired (staging according to Hamburger and Hamilton [11,12]).
- 2 Collect embryos into chilled chick saline as described [13], remove embryos from the vitelline membrane, then remove yolk and blood by rinsing several times in cold chick saline. Extra-embryonic membranes and large body cavities (pericardial sack for stages HH12–18; pericardial sack, brain vesicles, atria, allantois, eye for embryos older than HH19) should be opened to minimize trapping of reagents. Fix embryos overnight at 4°C in freshly prepared 4% paraformaldehyde.
- 3 Rinse embryos in PBS, then in PBTw, and dehydrate in a graded methanol series 25%, 50%, 75%, 100%, 100%) before cooling to –20°C overnight (or up to 7 d). Although some ISH protocols indicate that embryos can be stored for several months in methanol, we have found that storage longer than 7 days reduces hybridization signal (see Note 3).
- 4 Rehydrate by reversing the methanol series (75%, 50% 25% MeOH, PBS). Rinse embryos twice in PBS, sort by stage and treat older embryos with proteinase K: stages HH8–13 and 14–18 at 10 µg/mL of proteinase K for 10 and 20 min, respectively; stages HH19 and older at 20 µg/mL of proteinase K for 20 min. Individual lots of proteinase K vary in effective concentration. New lots should be tested over a concentration range to identify the optimal

²DIG labeled LNA probes are used at a final concentration of 5nM and hybridized at 22°C below the calculated probe:RNA duplex melting temperature. The hybridization temperature can be varied by a few degrees to accommodate different melting temperatures when more than one probe is used. Successful hybridizations are obtained over a relatively narrow probe concentration window (see Darnell et al [7]).

³Although some ISH protocols indicate that embryos can be stored for up to several months in methanol, we have found that storage in methanol for longer than 7 days significantly reduces hybridization signal for detection of both mRNAs and miRNAs.

conditions for proteinase K treatment. Rinse embryos repeatedly with PBTw to stop the digestion.

- 5 Fix embryos in 4% paraformaldehyde/0.1% Glutaraldehyde in PBTw for 30 min at room temperature. Rinse embryos 2x briefly with PBTw. If low detection signal is obtained, try eliminating this step.
- 6 Remove PBTw, add 1ml of prehyb solution and allow embryos to sink. Replace with fresh prehyb solution. Embryos can be stored for fewer than 7 d in prehyb solution at -20°C .

3.3 In situ hybridization

- 7 Sort embryos into pools for specific probe reactions. Place embryos for a given hybridization reaction in separate vials or in wells of a 24 well plate.
- 8 Prehybridize at the hybridization temperature (22°C below the predicted melting temperature as indicated by the supplier) for 2 hours on a gently shaking or nutating platform in an incubator, or if using glass vials, in a shaking water bath (see Note 2).
- 9 Add $1\mu\text{l}$ of the $5\mu\text{M}$ working probe stock to each ml of prehyb. Final probe concentration is 5nM .
- 10 Carry out hybridizations in small glass vials or in 24-well plates in a shaking hybridization oven or water bath for 48 hours at 22°C below the calculated melting temperature of the single or mixed LNAs. Overnight hybridization is often effective for abundant mRNAs, however longer hybridization times will give progressively higher signal (see Note 4). For long hybridizations, wells or tubes should be tightly sealed to prevent evaporation. Up to a 5°C spread in annealing temperature ($20\text{--}25^{\circ}\text{C}$ below the melting temperature) is consistent with hybridization [10]. This is useful if you are hybridizing with multiple probes for a single mRNA.
- 11 Prewarm washing solutions ($2\times\text{SSC}$, $0.2\times\text{SSC}$) to hybridization temperature before beginning washes. Adding colder washing solutions leads to high background.
- 12 Remove hybridization solution containing probe (probe solutions can be saved and reused) and replace immediately with hot (hybridization temperature) $2\times\text{SSC}$ washing buffer. Washes can be done in vials or in 6 well plates with 24 mm Netwell Inserts.
- 13 Larger washing volumes are important for removal of all unbound LNAs. Plates and inserts help maximize wash volume and minimize embryo handling and damage.

⁴Although overnight hybridizations can be sufficient, increasing the hybridization times (up to 5 days) will give progressively higher signal. For long hybridizations, wells or hybridization tubes should be tightly sealed to prevent evaporation.

- 14 Repeat washing step for a total of 3x 20 minutes with 2xSSC washing buffer, followed by 3x 20 minutes with 0.2xSSC washing buffer (see Note 5).
- 15 Rinse 2x 10 minutes with KBTw at room temperature.

3.4 Antibody incubation

- 16 Transfer embryos into clean 24-well plates.
- 17 Block embryos in 20% sheep serum in KBTw for 2–3 hours, or longer at 4°C.
- 18 During this blocking period, preabsorb the anti-DIG-AP Fab fragment by adding 3mg chick embryo powder per 500ul of 1:500 dilution of antibody in KBTw containing 20% sheep serum. When ready to use, centrifuge to pellet the chick embryo powder.
- 19 Add an appropriate volume of the preabsorbed anti-DIG AP Fab fragment to embryos in 20% sheep serum in KBTw to achieve a 1:2000–4000 dilution. Incubate at 4 °C overnight on a nutator.
- 20 Remove antibody (solution can be saved and reused) and replace with KBTw. Optional: move embryos to 6-well plates with mesh well inserts for increased wash volumes.
- 21 Wash in KBTw on a shaker or nutator at room temperature 5x 1 hour (may continue overnight at 4°C).

3.5 Color Reaction

- 22 Rinse embryos twice for 10 min in NTMTw.
- 23 For color reactions, mix NBT/BCIP color reaction solution and switch this for NTMTw rinse. Carry out color reactions for 1–6 h at room temperature on a nutator until signal or background becomes visible, followed by overnight washing in KBTw at 4°C. Do a second or third round of color reaction until each probe has yielded strong signal (see Note 6). Volume sufficient to cover embryos completely is enough.
- 24 Permanently terminate labeling reactions by washing with PBS.
- 25 Dehydrate embryos through a graded methanol series to reduce background and enhance signal.
- 26 Rehydrate and store in PBS plus 0.1% sodium azide.

⁵Embryos should not be allowed to cool while being transferred from hybridization solution to the first hot wash or between hot washes. All wash buffers for these steps should be prewarmed.

⁶Many expression patterns are not detectable during the first few hours of color reaction. Signal to noise can be maximized by performing the first color reaction until background color becomes just visible, then returning the embryos to an over night wash in KBTw, followed by a second color reaction and washing cycle. This can be repeated daily for several days. Prolonged washing in KBTw following the last staining reaction can sometimes reduce residual background. Dehydration through a methanol series will intensify the staining color and help to remove background.

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Figure 1.

ISH localization of the cardiac troponin T mRNAs in stage 22 chicken (A) and stage 35 *Xenopus* (B) embryos. For each species, a single LNA probe containing two DIG molecules was used. Arrow in (A) points to intense labeling of the heart; arrowheads in (A) and (B) point to mRNA localization in the skeletal muscles of the myotomes. Cardiac Troponin T mRNAs were not detected in the *Xenopus* hearts at this stage.