In Situ Hybridization Detection of mRNAs and MicroRNAs on Tissue Sections With TSA Amplification

This protocol has been optimized for detection of mRNAs using antisense riboprobes, or microRNAs using LNA containing oligonucleotides (LNA probes). Riboprobes are generated according to standard procedures, while LNA probes can be ordered from Exiqon (www.exiqon.com). LNA probes can be purchased with a DIG at the 5' and /or 3'ends. We prefer to purchase LNAs that are DIG labeled at the 5' end, and then to add a 3' DIG using the Roche DIG Oligonucleotide 3'-End 2nd Generation Labeling Kit (cat# 03 353 575 910). LNA probes can also be tailed at the 3' end using the Roche DIG Oligonucleotide tailing kit, 2nd Generation (cat # 03353583910). This procedure generates a 3' tail of approximately 50nt containing several DIG molecules.

Important Note for MicroRNA Detection: This protocol was developed prior to the publication by Pena et al (2009) showing that combining paraformaldehyde and EDC fixation improves microRNA retention in tissue sections. Although we have not evaluated the EDC fixation protocol, the published images show significantly enhanced signal. EDC fixation has NOT been incorporated into the protocol below.

Reference: Pena JT, Sohn-Lee C, Rouhanifard SH, Ludwig J, Hafner M, Mihailovic A, Lim C, Holoch D, Berninger P, Zavolan M, Tuschl T. miRNA in situ hybridization detection using formaldehyde and EDC fixed tissues. Nat Methods. 2009 Feb;6(2):139-41. Epub 2009 Jan 11. PMID: 19137005

I. Fixation and Sectioning

1. Fix embryos or tissue samples in [6V EtOH 100% (30mls), 3V Formaldehyde 37% (15mls), 1V Acetic Acid 100% (5mls), 50mls total] from 1hr at RT to overnight at 4ºC, depending on embryo or tissue size.

   NOTE: Prepare fix fresh for each use. Fixation for longer than recommended times can reduce hybridization signal.

   NOTE: In our hands fixation in formaldehyde alone results in variable and reduced hybridization signal. This might result from loss of RNAs from the sections.

2. Dehydrate specimens with two changes in 70% EtOH, followed by two changes in 100% EtOH. For small embryos (up to chicken HH Stage 15, mouse TS 15 [E9.5]) or tissue samples, 10 min each is sufficient. For older embryos or larger tissue fragments, 15-30 min is recommended.

3. Wash 2 x 15 min in fresh xylene. For small embryos or tissue samples, 2 x 10 min is sufficient.

4. Drop embryos into 1:1 paraplast: xylene mix @ 60ºC for 1 to 1.5 hr, followed by 4 x 1 hr changes of fresh paraplast. For small embryos or tissue samples, skip the 1:1 paraplast: xylene step and go directly to 2 x 1 hr changes of fresh paraplast.

5. Transfer embryos or tissue samples to plastic sectioning molds containing paraplast @ 60ºC. This is best accomplished using hot specimen needles and a dissecting microscope to properly orient embryos or tissue samples. Allow paraplast to harden.

6. Cut 10-14um sections and place on Superfrost-plus slides.

II. Section Pretreatment

7 Dewax slides in fresh Histoclear 3 x 5 min (visually inspect sections to be sure that all paraplast has been removed). Transfer slides to 100% methanol for 5 min, then to methanol containing 0.3% H_20_2 for 20 min to quench endogenous peroxidase activity.

8. Transfer slides to 100% EtOH, then rehydrate through an ethanol series 2 min each at EtOH 95% 90%, 80%, 70%, 40%. Rinse 1 x 5 min in PBS (slides can remain in PBS for a short time if needed).

   NOTE: Be careful to not let sections dry between ethanol washes, as this can lead to nonspecific probe binding and splotchy in situs.

   NOTE: Histoclear is recommended to remove paraffin from sections; using xylene reduces hybridization signal.

9. Treat sections with 1µg/ml Proteinase K in PBS for 10 min at 37ºC. This step can be performed in a 37˚C water bath to ensure rapid temperature equilibration. These parameters work well for sections of chicken and mouse embryos, however other tissue types may require different conditions.

   NOTE: Proteinase K activity varies from lot to lot. It is important to determine the best concentration/time parameters for your lot.

10. Stop Proteinase K reaction by placing slides in 2mg/ml glycine in PBS for 2 min at RT.

11. Wash 1 x 5 min in PBS (slides can be left in PBS for a short time), then refix for 20 min at RT in 4% paraformaldehyde/PBS. This can be performed in a Coplin jar or slide mailer (see below).

12. Wash 1 x 5 min in PBS, then 1 x 5 min in 2X SSC at RT.

III. Prehybridization/Hybridization

13. Prehybridization: Remove as much SSC as possible by blotting the slide edge with a paper towel or Kimwipe, then transfer to hybridization solution without probe prewarmed to hybridization temperature (65-70ºC for antisense probes, or 22ºC below predicted Tm for LNA probes) for 1-2 hrs. This can be accomplished using a slide mailer or other small chamber. If necessary, slides can be left in pre hyb solution overnight.

14. Hybridization: Remove slides from prehyb and drain off as much solution as possible; dry the back of the slide. Lie slides flat and pipet 200µl (more or less) of hybridization solution onto slides containing 100ng/ml of Digoxigenin-labeled riboprobe, or 5nM (5 picomoles/ml) of LNA oligo. Carefully place a plastic coverslip over the hybridization solution, avoiding bubbles. Incubate overnight at 65-70ºC for antisense probes, or 22ºC below predicted Tm for LNA probes, in a humid chamber containing Whatman paper drenched with 50% formamide/2x SSC.

Alternative Method: Place slides in slide mailers containing hybridization solution plus probe. Seal top with plastic wrap (parafilm melts), and place upright in humid chamber with formamide/SSC. Although mailers require more probe and hyb mix (13-15ml), mailers-hyb solution can be stored at –20˚C and reused.

NOTE: Be sure to evenly distribute the hybridization solution on the slide. This can be accomplished by gently rocking the slide after adding the hyb solution and prior to coverslipping.

NOTE: LNA probe concentrations can be varied up to ten fold higher or lower, however in general higher concentrations are not better and can lead to high background. Riboprobes can also be used over a five to ten fold concentration range, although in our hands 100ng/ml is optimal for most probes.

IV. Slide Washing

15. Place slides with coverslips in 50% formamide/ 1x SSC/ 0.1% Tween-20 prewarmed to hybridization temperature. Wash for 15 min at 65ºC. Coverslips should float off of the slides during this wash; remove. Mailers do not work well for washing; we use standard glass slide staining dish.

16. Perform 4-5 washes of 30 min each at hybridization temperature in 50% formamide/ 1x SSC/ 0.1% Tween-20 (washes can be extended up to 2hrs each).
17. Wash 2-4 x, 30 min each, in TNT (Tris-NaCl plus 0.1% Tween-20) at RT (washing times can be extended).

V. Digoxigenin Immunohistochemistry and TSA Amplification

18. Block sections for 30 min in TNB (0.5g block reagent/100ml TN) at RT. This can be accomplished in slide mailers or by placing 800ul of solution on slide in a humid chamber, without a coverslip.
19. Add 500µl of blocking mix containing a 1/500 dilution of anti-digoxigenin-POD (Roche) directly to the existing block solution already on the slide. Mix carefully to distribute evenly. Coverslip with parafilm cut to size and incubate for 2 hrs at RT. This step can also be accomplished in slide mailers.

    NOTE: Preabsorb antibody as follows: For 2 mls of final diluted antibody solution, place 400ul antibody in 0.5ml of TNB. Add 3mg of chicken embryo powder, shake gently at 4ºC for 2 hours to overnight. Spin, remove supernatant and dilute to 2ml with TNB.

20. Wash 4 x 10 min in TNT at RT with agitation (these washes can go longer or overnight).
21. Transfer slides to a humid chamber (face up, wipe back of slide dry). Pre equilibrate sections by adding 50ul of 1X Amplification Diluent to each slide, mixing with the TNT remaining on the slide, and then removing.
22. Pipet 200ul of Fluorophore Tyramide (Amplification Reagent) Working Solution onto each slide. Incubate slides at RT for 10 min. TSA amplification uses the PerkinElmer TSA Plus Fluorescent System (Cat # NEL760).

    NOTE: Prepare Fluorophore Tyramide Working Solution by diluting the Fluorophore Tyramide Stock Solution 1:100 using 1X Plus Amplification Diluent. Approximately 200-300ul is required per slide. All components needed for the Fluorophore Tyramide Working Solution are provided in the TSA Plus Florescence Systems Kit (PerkinElmer).

23. Transfer slides to a coplin jar or slide staining dish and wash 4 x 5 min in TNT at RT with some agitation.

    NOTE: The TSA procedure deposits multiple Tryamide-Fluorescein conjugates (or other fluors) in the immediate vicinity of HRP. At this point you have completed a fluorescence in situ hybridization. If desired, you can check for fluorescence signal using a fluorescence microscope. The following steps convert the fluorescence signal to a visual reaction product using alkaline phosphatase conjugated anti-Fluorescein.

24. Transfer slides to a humid chamber and pipet 500ul of a 1/500 dilution of Anti-Fluorescein-AP in TNB onto each slide. Incubate for 2 hrs at RT.
25. Wash 4 x 5 min in TNT at RT with agitation (these washes can go longer or overnight).
26. Wash 2 x 5 min in NTMTw (longer washes are OK).
27. Perform color reaction at RT using NTMTw containing 4.5µl NBT and 3.5µl BCIP per ml. For probes that generally give a strong signal by whole mount, signal on sections can appear within 1-3 min, and most probes show some signal by 30 min of incubation. The color reaction can proceed up to several hours if necessary.
28. Stop the reaction by placing slides in KTBT. Slides can be stored overnight and the color reaction repeated if necessary.

29. Once the color reaction has proceeded to your satisfaction, rinse slides in PBS (this will permanently stop the reaction). Optional: refix for 20 min in 4% paraformaldehyde in PBS, then wash in PBS.

30. Just prior to mounting, wash briefly in DI H_20. Drain slide to remove most of the water (do not allow to dry), then coverslip using an aqueous based mounting medium such as AquaPolymount.

    NOTE: Xylene and Toluene based mounting media are not recommended as they cause the reaction product to crystallize.

Solutions and Reagents

Slide Mailers: Cyto-tek Slide Mailers are made by Sakura Finetek (part #: 4310) and are available from VWR, Cat#: 25608-874 and Fisher, Cat#: NC9574746. Mailer capacity is 15-20ml.

Prehybridization/Hybridization Solution

	Final concentration	**For 10 ml**	**For 50 ml**
10x salts	1x	1 ml	5 ml
Formamide	50%	5 ml	25 ml
50% dextran sulfate	10%	2 ml	10 ml
Yeast RNA (10mg/ml)	1 mg/ml	1 ml	5 ml
50x Denhardt's Solution	1x	200 µl	1 ml
H2O		800 µl	to 50 ml

10x salts: (200 ml)

NaCl	22.8 g
TrisBase	0.268 g
TrisHCl	2.8 g
NaH2PO4.2H2O	1.56 g (or 1.35 g for NaH2PO4.H2O or 1.14 g for NaH2PO4)
Na2HPO4	1.42 g
0,5M EDTA	5 ml
H20 to 200 ml	

TNT Wash Buffer: (1 Liter)

1M TRIS-HCl, pH 7.5	100ml
5M NaCl	30ml
Tween-20	1ml
H20 to 1000 ml	

TNB Blocking Buffer: (500 ml)

1M TRIS-HCl, pH 7.5	50ml
5M NaCl	15ml
Blocking Reagent	1g
H20 to 500ml	

NTMTw:	50ml	200 ml
NaCl 5M	1 ml	4 ml
Tris 2M pH 9.5	2.5 ml	10 ml
MgCl2 2M	1.25 ml	5 ml
Tween-20	500 µl	2 ml
H20	to 50ml	to 200ml

KTBT:

50 mM Tris-HCl (pH 7.5)	10 ml of 1M stock
150 mM NaCl	6 ml of 5M stock
10 mM KCl	1 ml of 2M stock
1% Triton X-100	10 ml of 20% stock
MilliQ water	173 ml
total	200 ml

Proteinase K: Proteinase K, Molecular Grade, 100mg. Sigma # p2308

Blocking Reagent: Roche Cat. # 11 096176001

Slides: Microslides, Superfrost Plus. VWR Cat. No. 48311-703

Anti-Digoxigenin-AP, Fab Fragment: Roche Cat. # 11093 274 910 (150U/200ul)

NBT: Sigma Cat # N6876. Prepare a stock solution of 75mg/ml in 70% Dimethyl formamide (DMF)

BCIP: Sigma Cat # B8503. Prepare a stock solution of 50mg.ml in 100% DMF

DMF: N, N Dimethyl-formamide; Sigma Cat # D-8654, 500ml.

TSA PLUS FLUORESCENCE SYSTEM Kit: PerkinElmer Catalog # NEL760.

AquaPolyMount: PolySciences, Inc. Cat # 18606, 20ml.

Chick embryo Powder:
Homogenize day 3-5 chick embryos in a minimum volume of PBS. Add 4 volumes of ice- cold acetone, mix and ice for 30min. Spin at 10,000 g for 10 min; remove supernatant, wash pellet with icy 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.

This protocol was prepared by the GEISHA In Situ Hybridization Project (http:geisha.arizona.edu).
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