

In situ hybridization on mouse paraffin sections

Modified by Ellen Chang 2009

from protocols by Hiroki Kuroda, Lise Zakin and Eddy De Robertis

Notes: This protocol is designed for embryos 10.5 dpc to 14.5 dpc.

Dissections and fixation

- Dissect embryos in 1X PBS
- Fix embryos in 4% PFA in PBS o/n at 4°C

Embedding and sectioning

- Wash 3x in 1X PBS for 5 minutes
- Wash in 70% EtoH for 1 hour
- Wash 1x in 95% EtoH for 1 hour
- Wash 3x in 100% EtoH
- Wash 3x in toluene
- Wash 3x for 1 hour in filtered paraffin (Mc Cormick scientific cat# 502004) in an oven at 75°C
- Wash 1 hour in paraffin inside a vacuum oven at 75°C to prevent paraffin from solidifying
- Embed embryos in fresh paraffin
- Allow the blocks to solidify at least 24 hours
- Cut 10µm thick sections using a microtome

DAY ONE

Rehydration (in fume hood)

- 2x xylene, 5 minutes each
- 2x 100% EtOH, 1 minute each
- 1x 70% EtOH, 1 minute
- 1x 2X SSPE, 5 minutes

Pretreatment

- Refix with 1ml per slide 4% PFA in PBS at RT for 15 minutes
- Rinse with 2X SSPE for 5 minutes
- Using humidified chamber, incubate slides in 1 ml per slide Proteinase K (3 µg/ml PBS) at 37°C for 30 minutes
- Rinse slides in 2X SSPE for 5 minutes
- Incubate slides in 1 ml/slide 0.2M HCl at RT for 15 minutes
- Rinse with 2X SSPE for 5 minutes

Hybridization

- Dilute DIG labeled RNA probe in hybridization buffer (0.1-1µg/ml). Denature the probe mix 5-10 minutes at 70°C. Calculate 100µl per section
- Add probe mix to each slide and cover slide with a coverslip (use 24x60mm number 1 coverslips)

-Hybridize o/n at 65°C in a sealed plastic slide box with 2 sheets of whatman paper wetted with 0.2X SSC + 50% formamide to prevent slides from drying

DAY TWO

Post-hybridization washes

- Transfer slides to a slide rack (one without a central support to allow the coverslips to fall off the slides) immersed in 200-300ml washing solution (enough to cover the slides) pre-warmed at 65°C. Perform the washes in a water-bath placed inside a fume hood (to avoid inhalation of the warm formamide present in the washing solution)
- Wash 1x 15 minutes at 65°C in washing solution to allow coverslips to fall off
- Wash 2x 30 minutes at 65°C in washing solution
- Wash 2x 30 minutes with 1X MABT at room temperature

Blocking and antibody staining

- Remove slides from the slide rack and place in a humidified chamber
- Block at least 1 hour in 1X MABT + 2% Blocking reagent + 20% heat inactivated sheep serum (goat serum works as well) at room temperature (no coverslips)
- Incubate o/n at 4°C with fresh 1X MABT + 2% Blocking reagent + 20% heat inactivated sheep serum containing a 1/2000 dilution of anti-DIG alkaline phosphatase antibody (Roche cat# 11093274910) in a humidified chamber. Use 100 µl antibody solution per section and coverslip

DAY THREE

Post-antibody washes

- Transfer slides to a slide rack (one without a central support to allow the coverslips to fall off the slides) immersed in 200-300ml 1X MABT (enough to cover the slides)
- Wash 4-5x 30 minutes each in 1X MABT

Staining reaction

- Reveal in humidified chamber protected from light, with 1 ml per slide BM purple AP substrate (Roche cat# 11 442 074 001) for 1-24 hours at 4°C or room temperature depending on the quality of the probe
- Wash 3x 5 minutes with PBS
- Dehydrate and mount slides in permount for photography

SOLUTIONS

4% PFA in PBS: mix 4g paraformaldehyde (PFA) with 100ml 1X PBS. Place on a heating block until solution becomes clear, immediately filter into a bottle placed on ice aliquot and freeze. Aliquots can only be thawed once

20X SSPE: for 1 liter, 175.3g NaCl, 27.6g NaH₂PO₄, 7.4g EDTA. pH to 7.4

Proteinase K RNA grade: Invitrogen cat# 25530-049

10X Salt: 2M NaCl, 100 mM Tris-HCl pH 7.5, 100mM phosphate buffer pH 7.4, 50 mM EDTA pH8

100 ml 5M NaCl
25 ml 1M Tris-HCl pH7.5
25 ml 0.5M EDTA
19.35 ml 1M Na₂HPO₄
5.65 ml 1M NaH₂PO₄
H₂O QSP 250 ml

100X Denhardt's: stored aliquoted at -20°C

2g bovine serum albumin
2g FicollTM
2g polyvinylpyrrolidone
H₂O QSP 100 ml

Hybridization buffer: store 1-2 ml aliquots at -20°C

1X salt
50% formamide
10% dextran sulphate
Torula RNA 1 mg/ml (Roche/Sigma cat# 10109509001)
1X Denhardt's

Washing solution: 1X SSC, 50% formamide, 0.1% Tween20

1X MABT: 100 mM maleic acid, 150mM NaCl, 0.1% Tween20, pH 7.5

5X MAB:

21.91g NaCl
29.02g Maleic Acid
pH to 7.5 with NaOH: first add 18g NaOH pellets, then adjust pH with concentrated NaOH solution
H₂O QSP 500ml

Blocking reagent: Roche cat# 11 096 176 001. Make 10% stocks in 1X MAB (no tween20) and store at -20°C