Colorimetric and Florescent In Situ Hybridization

Modified from Mariani lab

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Day1

1. Deparaffin slides

Xylene	5min
Xylene	10min
100% EtOH	5min
95% EtOH	5min
70%EtOH	5min
1XPBST (0.1%tween-20)	5min

2. Permeabilization
   5min incubation with 7.5ug/ml proteinase K (in PBST) (on slide)
   5min wash in PBST in a Jar (1)
   Postfix in 4% PFA with 0.2% Glutaraldehyde for 20min (on slide)
   (add 8ul 25% glutaraldehyde in 1ml 4% PFA)
   5min wash in PBST Jar (1)

3. Hybridization
   a. Pre-hybridise slides in **preheated** hybridization buffer (HYB buffer) at 68C for (at least) 1h in a Jar (2).
   b. Dilute the RNA probe to 10ng-20ng/ul with HYB buffer, heat on the heat board at 80C for 10min, and then put directly on ice. Apply 100ul probe per slide. Cover with a cover slide and then cover with a piece of para film.
   c. Put slide in a humidified chamber with filter paper soaked with H2O or HYB buffer at the bottom. Wrap the chamber if necessary. Hybridise with probes overnight (12-16 hours).
      HYB buffer (200ml):
      100ml Formamide, 50ml 20XSSC (ph 7.0), 1g Torula Yeast (tRNA) (**R6625 SIGMA)**, 10mg Heparin Sodium Salt (50ug/ml), 50ml H2O.
      20X SSC (500ml):
      87.65g Sodium Chloride, 44.1g Sodium Citrate, dissolved in H2O and adjust the pH to 7.0 with Citric acid and bring the solution to the final volume of 500ml.

Day 2 (Florescent)

4. Stringency washes
   a. Wash slides in **preheated** wash buffer at 68 1X15min, 2X30min in a jar (2)

   Do not remove the cover-slide from the slide. Place the slide with the cover-slide into the jar and let the cover0slide detach from the slide in the buffer.

   Wash buffer (200ml)

   100ml Formamide, 10ml 20XSSC, 200ul Tween-20, 89.8ml H2O.

   b. Wash slide in TNT buffer 3X15min at room temperature in a Jar (3).

   TNT (1 litter): 100ml 1M Tris pH7.5, 30ml 5M NaCl, 1ml 100% Tween-20, 869ml H2O.

5. Blocking endogenous Peroxidase

   Incubate in 2% H2O2 diluted in TNT for 30-60min at RT in a Jar (3).

   Add 2ml of 30%H2O2 to 30ml TNT.

   Wash in TNT for 1X5min in a Jar (3).

6. Blocking in 2% Roche Blocking Reagent (11096176001 Roche, in 1XTNT) for at least 1h on slide at RT.
7. Antibody incubation.

   Dilute antibody with 2% Blocking Reagent: (start with the strongest probe)

   Anti-DIG-POD 1/500 (11207733910 ROCHE, Anti-Digoxigenin-POD, Fab fragments)

   Anti-DNP-POD 1/200 (Perkin-Elmer Cat# NEL747A001KT)

   Anti-Fluo-POD 1/200 (Invitrogen cat# A-21253)

   Incubate at 4C overnight (on slide). Cover the section with a piece of para film.

Day 3 (Florescent)

8. Color reaction
   a. Wash 3x5 min in TNT in a Jar (3)
   b. Equilibrate sections for 5min with Perkin Elmer Amplification Diluent (supplied with the kit) (on slide) (TSA Cyanine 3 and Fluorescein system: Perkin Elmer Cat# NEL753001KT)
   c. Prepare appropriate dilution of the fluorophore in amplification diluent. (1:100 in amplification diluent)

   d. (the following steps should be prepared in dark to avoid bleaching)

   Apply 100ul of antibody per slide; incubate for no more than 1h at RT. Cover the section with a piece of para film.

   e. Wash in TNT 1x5min in a Jar (3)
   f. Quench reaction by washing in 2% H2O2 in TNT for 30-60min. (If this step is incomplete, you would get cross-reaction with the following staining. Can skip if you only have one color)
   g. Wash in TNT 1x5min in a Jar (3).
   h. For developing the next color, repeat step 6-8 .
9. Nuclear counterstain: counterstain with DPAI (300nM) for 5min at RT, wash with 1X5min PBS in a Jar (4).

10. Mounting with mounting medium.

Day 2 (Colorimetric)

4. Stringency washes
   a. Wash slides in **preheated** wash buffer at 68 1X15min, 2X30min in a jar (2)

   Do not remove the cover-slide from the slide. Place the slide with the cover-slide into the jar and let the cover0slide detach from the slide in the buffer.

   Wash buffer (200ml)

   100ml Formamide, 10ml 20XSSC, 200ul Tween-20, 89.8ml H2O.

   b. Wash slide 3X15min in MABT in a jar (3)

      MABT (1litter): 200ml 5XMAB, 1ml 100% Tween-20, 799ml H2O

      5X MAB: 500mM Maleic Acid, 150mM NaCl, adjust to pH7.5 with NaOH.

5. Blocking in 2% Roche Blocking Reagent (11096176001 Roche) for at least 1h on slide at RT.
6. Antibody incubation.

   Dilute antibody with 2% Blocking Reagent

   Anti-DIG-AP 1/2000 (11093274910 ROCHE, Anti-Digoxigenin-AP, Fab fragments)

   Incubate at 4C overnight (on slide). Cover the section with a piece of para film.

Day 3 (Colorimetric)

1. Color reaction
   a. Wash 3x5 min in MABT in a Jar (3)
   b. Wash in staining buffer NTMT 2X5min in a Jar (4)

      NTMT(100ml): 10ml 1M Tris pH9.5, 5ml 1M MgCl2, 2ml 5M NaCl, 100ul 100% Tween-20. 82.9ml H20. The Color reaction is highly dependent on the pH. Make sure the pH of the buffer is 9.5.
   c. Apply staining solution BM purple to each slide. Cover the section with a piece of para film. Keep the slide in dark for 2h-overnight at RT. After one day, you can change the staining solution. Wash the slide in NTMT and they apply fresh staining solution.
   d. Wash 3X5min in PBST.
2. Counterstaining

   15 dips in H2O

   1min incubation in nuclear fast red

   15 dips in H2O

   10min incubation in H2O in a Jar (4)
3. Dehydrate with 2X2min 95% EtOH

   2X2min 100 EtOH

   2X2min Xylene

   Mounting with Cytoseal 60 and cover slide.