In situ hybridization on Sections - Hiroki Kuroda

(1st day)
Note. Embed sample in paraffin in HE staining case.
1. Cut samples at 20-25 µm thickness
2. Dry slides for overnight at 45ºC.

(2nd day)
1. Rehydrate
   (Xylene for 5 min) x2
   (100% EtOH for 1 min) x2
   70% EtOH for 1 min
   2 x SSPE
2. Refix in 4% Paraformaldehyde in PBS at RT for 15 min.
3. Rinse with 2 x SSPE
4. Incubate slides in Proteinase K (3 µg/ml in PBSw) at 37ºC for 30 min.

Note: This step is for making RNA more accessible to hybridization.
5. Rinse slides in 2x SSPE
6. Incubate slides in 0.2M HCl at room temperature for 15 min.

Note: This step is to denature proteins, nick DNA, and partially reverse the fixation step.
7. Rinse with 2x SSPE.
8. Add 400 µl of hybdidization buffer to each slide and incubate in a humid chamber at 65 ºC for 2 hours.
9. Drain off excess hybridization buffer.
10. Add 110 µl of 0.5 µg/ml probe solution to each slide.
11. Put glass coverslips on the top.
12. Incubate slides in a humid chamber at 65ºC overnight.

(3rd day)
1. Soak slides in 2x SSPE until the coverslips fall off (in a Coplin jar).
2. Add enough hybridization buffer to cover the slide and incubate at room temperature for 5 min.
3. Drain slides and add 50 % hybridization buffer: 50% 2x SSPE: 0.3% CHAPS.
4. Incubate at RT for 10 min.
5. Soak in 2x SSPE for 20 minutes (in a coplan jar).
6. (Rinse slides in PBSw for 10 min in a coplan jar) x3
7. Add 500 µl of Antibody Buffer to each slide.
8. Incubate for 2 h at RT.

Note: At the same time, pre-block the antibody (anti-dig AP fab fragments, diluted :1000) in the Antibody Buffer at 4ºC, rocking for 2 hours.
9. Drain slides and add 200 µl of pre-blocked antibody and incubate at RT for 1 h.
10. (Rinse slides in 0.1 % BSA in PBSw for 10 min in a coplan jar) x3.
11. Rinse slides in AP buffer for 10 min in a coplan jar.
12. Begin staining by 10-fold diluted BM purple with AP buffer and incubate at 4ºC in the dark until the strength of staining is appropriate..
13. Wash in PBSw and then stop the reaction with Stop Solution for 15 min.

Solutions:

10x PBS: 80g NaCl, 2g KCl, 14.4g Na2HPO4, 2.4g KH2PO4, pH to 7.4,. Adjust volume to 1L with DDW, DEPC treat and autoclave.

PBSw: PBS with 0.1% Tween-20

20x SSPE: 175.3g NaCl, 27.6 g of NaH_2PO_4, 7.4 g of EDTA, pH 7.4, adjust volume to 1L with DDW, DEPC treat and autoclave.

Hybridization Solution: Make 1L, filter, and store at 20ºC in aliquots. (1st step) 10g Boehringer Block, 500ml Formamide, 250ml 20x SSC, Heat at 65 ºC for 1 hour. Add 120ml DEPC treated water, 100ml Torula RNA (10mg/ml in water; filtered), 2ml Heparin (50mg/ml in 1x SSC), 5ml 20% Tween-20, 10ml 10% CHAPS, 10ml 0.5M EDTA.

Antibody Buffer: 10% Heat Inactivated Goat Serum,1% Boehringer Block, 0.1% Tween-20 Dissolve in PBS at 70 ºC, vortexing frequently, and then filter (0.45 µm).

AP buffer: Put 5 ml of 1M NaCl, 1M Tris, pH 9.5 and 5 ml of 0.5M $MgCl_2$ into 40 ml of DDW.
Note. Do not mix at high concentration, or precipitate will appear.

Stop Solution: 100mM Tris pH7.4, 1mM EDTA

Product:
Boehringer Block - Roche #1096176
Proteinase K - Gibco #25530-049
Anti-Dig-AP - Roche #1093274
BM purple - Roche