DIG in situ

I. *Probe preparation*

1. Digest 10 ug plasmid DNA with the appropriate enzyme
2. Check a small aliquot of the reaction to check the completion of the digestion
3. Purify the linearized plasmid using the QIAquick Gel Extraction Kit (Qiagen)

II. *Synthesis of the riboprobe(all components from LaRoeche)*

1. Reaction mix: 1 ug purified linearized plasmid
   2 ul 10X transcription buffer
   2 ul 10X NTP-labelling mixture
   1 ul Rnase inhibitor
   2 ul RNA polimerase (SP6, T3 or T7)
   DEPC-treated dest. water up to 20 ul

Reaction time: 90 min at 37°C
To check the reaction load 1-2 ul from the mixture to 1.5-2% agarose gel and run shortly. The riboprobe should be visible on EtBr stained gel.

2. Add 2 ul RNase free DNase to the reaction mix and incubate 15 min at 37°C.
3. Add 2.5 ul NaAc (pH 5.2) and 100 ul Ethanol to precipitate the probe (incubate 30 min at –80°C)
4. Redissolve the pellet in 50-100 ul DEPC-treated water and store at –80°C for up to 1 year.

(Optional: the riboprobes work well up to 600 bp. If the probe longer than 600 bp, a partial hydrolysis should perform to reduce the length).

III. *Tissue processing*

We usually fix the tissue with alkaline fixative which significantly increase the signal intensity.

1. Fix the tissue overnight at 4°C in 4%PFA/TBS (50 mM Tris-HCl, pH 9.5, 150 mM NaCl)
2. Process for paraffin embedding.

IV. *Section pretreatments:*

1. Dewax slides 3 x 10 min in xylene and ethanol series
2. Postfix in 4%PFA/TBS (pH 9.5) for 20 min
3. Rinse 3-5 times in TBS (pH 7.5!)
4. treat sections for 10 min with 200 mM HCl
5. rinse 3-5 times in TBS (pH 7.5)
6. acetylate for 10 min in freshly prepared 0.5% acetic anhydride in 100 mM Tris-HCl, pH 8.0 (optional)

7. treat sections for 20-30 min in 10 ug/ml protK in TBS containing 2 mM CaCl2 at 37°C.
8. 2 times 5 min in TBS
9. dehydrate sections in ethanol series
10. rinse briefly in cloroform (optional)
11. place slides onto a heating plate at 55°C until the preparation of the hybridisation mixture.

V. Hybridization

1. Dilute probe in hybridisation solution (2% SSC, 50% formamide, 10% dextran sulfate, 0.02% SDS, 0.01% sheared salmon sperm DNA). Incubate the mixture at 85°C for 5 min.
2. add about 50 ul probe on section
3. put the slides with the hybmix for 1 min an a 95°C heating plate (this step improve the signal/background ratio. However, bone sections can easily detach at this step. If this is the case, omit the this step.
4. cover sections with parafilm
5. incubate in humid chamber ON at 55°C.

VI. Washes and Detection of the signal

1. Incubate the sections in 2x SSC to remove the parafilm
2. wash sections 3 x 30 min at 55°C with 50%formamide/1xSSC
3. wash 3 x 20 min at RT with 1xSSC
4. rinse 3 times in TBS (pH 7.5)
5. block the sections with 10% sheep serum/TBS/0.1% Tween 20 for 1 hour
6. incubate for 60-120 min with AP-conjugated anti-DIG AB (La Roche) in the blocking solution (dilution 1:500)
7. Rinse 3 times in TBS
8. incubate the slides in DIGIII buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM MgCl2)
9. prepare the staining solution: 200 ul NBT/BCIP stock (La Rouche) in 10 ml DIG III
10. cover the sections with the staining solution and stain at RT in humid chamber
11. stop the reaction in TE buffer for 30 min
12. Mount with Aquatex