Whole mount in situ hybridization with DIG-labeled probes from Dennis O'Leary 2004

Unless otherwise noted,
Wash=5 min
Rinse=briefly, until the brains sink

Use RNAse free reagents and plastic wares until you finish hybridization

<Solutions to make>
PTW: PBS with 0/1% Tween-20
Hybridization mix
(for 50ml) Formamide 25ml, 20x SSC 3.25ml, 0.5M EDTA 0.5ml, 20mg/ml yeast RNA 125ul, 10% Tween-20 200ul, 50mg/ml Heparin 100ul, fill up with H2O to 50ml
10x TBST:
(for 100ml) NaCl 8g, KCl 0.2g, 1M Tris-HCl pH7.5 25ml, Tween-20 10 ml, fill up with H2O to 100ml
Make 1x solution on the day of use
NTMT:
(for 50ml) 5M NaCl 1ml, 2M Tris-HCl pH9.5 2.5ml, 1M MgCl2 2.5ml, 10%Tween-20 5ml, fill up with H2O to 50ml and add 10 drops of levamisol

Make from stocks on the day of use

1. Pretreatment of the brains
   A. Cut out the heads of embryos or pups and put them in ice-cold PBS (DEPC-treated)
   B. Take out brains
   C. Remove meninges using Jewelers forceps in both hands
      (I use a very sharp one in the right hand to remove the meninges and not so sharp one in the left mainly to hold the brain. I am right-handed)
      For cortex, this is easiest for E16.5 and E18.5, and removing meninges postnatal brains is difficult
   D. Fix the brain overnight at 4c in 4% PFA/phosphate buffer

2. Prehybridization
   A. Wash the brains twice in PTW
   B. Wash once with 50% methanol/PTW, then 100% methanol twice (You can store the brains at this point in –20c. for less than one month)
   C. Rehydrate brains through 75%, 50%, 25% methanol/PTW (allowing brains to settle), and wash twice with PTW
   D. Treat with 10 ug/ml proteinase K in PTW for 15-45 min.
      (The time needs to be adjusted for each probe and also the size of the brain. I used 30 min for Lhx2 probe on E18.5 mouse neocortex)

E. Remove proteinase solution, rinse briefly (be gentle) with PTW, and post-fix for 20min in 4% PFA/0.1% glutaraldehyde in PTW
F. Rinse and wash once with PTW. Transfer brains to 2ml round-bottom tubes (with screw-cap).
G. Rinse once with 1:1 PTW/hybridization mix. Let brains settle
H. Replace with 1ml hybridization mix and prehybridize at 70c for more than 1h (or the brains can be stored in prehybridization mix at –20c until you get ready for hybridization)

3. Hybridization
   A. Replace with hybridization mix with probe (concentration to try first=3 ug/ml).
   B. Incubate at 70c overnight (mix after 20-30 min)

4. Wash
   A. Rinse twice with pre-warmed (70c) hybridization mix
   B. Wash 2x30 min at 70c with pre-warmed hybridization mix
   C. Wash 20 min at 70c with pre-warmed 1:1 hybridization mix/TBST
   D. Rinse 3 times with TBST
   E. Wash 2x30 min with TBST
   F. Block brains with 10% heat-inactivated sheep serum in TBST, 3h
   G. Replace with anti-DIG antibody (1:5000, Roche) and rock overnight 4c

5. Color reaction
   A. Rinse 3 times with TBST.
   B. Wash 3x1h with 10 ml TBST, by rolling
   C. Wash 2x10 min in with NTMT
   D. Incubate with 1.5ml NTMT with 3.3ul/ml NBT and 3.5ul/ml BCIP
   E. Rock for the first 20 min, and then transfer to 24-well dish for observation
   F. The color may come after 30 min or not be apparent when you leave for home. If you stop, wash 3x with PTW and store in PTW plus 10 mM EDTA and 0.1% azide. If you don't, leave the brains in the cold room. During the color reaction, keep the brains in the dark as much as possible.

Supplements

1. **Making of riboprobes**
   A. Digest 10 ug of plasmid DNA with an appropriate restriction enzyme for >2h in 100 ul Rx
   B. Run 5 ul and make sure the digestion is complete.
   C. Ethanol precipitate after phenol/chloroform and chloroform treatment
   D. Dissolve in 20 ul of H_2O (I use filtered milli Q water rather than DEPC-treated water because DEPC is thought to be a potent inhibitor of RNA polymerase). The final concentration of the linear plasmid would be 0.5 ug/ul
   E. Make 50ul reaction of in vitro transcription

x ul H_2O (filtered milli Q)
10 ul 5x RNA polymerase buffer (Promega)
5 ul 100mM DTT (Promega)
5 ul plasmid (2.5 ug)
5 ul DIG labeling mix (Boehringer)
1 ul Ribonuclease inhibitor (labeled HRI on the white top, Amersham)
y ul RNA polymerase (T3, T7, or SP6, 80 units, Promega)

F. Incubate at 37˚C for 2h
G. Add 4 ul of RNase-free Dnase (Promega) and incubate for 15 more minutes
H. Add 6 ul of 4M LiCl and 150 ul of EtOH and precipitate
I. Dissolve in 100 ul DEPC-treated H_2O
J. Run the 1% agarose gel with known amount of RNA (I use previously made riboprobes with roughly known concentration but it can be a pretty rough estimation).
K. Dilute to convenient concentration (I usually get approximately 20 ug in total)
L. Store at -20˚C or -70˚C (some people prefer to dissolve in hybridization buffer but I do not).

2. Necessary reagents

A. DEPC-treated MilliQ water (add 4ml of DEPC for 2L water, shake, and autoclave for 40min)
B. DEPC-treated PBS (treat 10x PBS in the same way you treat H2O)
C. 4% PFA/PB(S) (made from 20% stock solution dissolved in distilled water) I don't worry about DEPC treatment of this
D. Proteinase K (EM Science 34568-2) Make 10mg/ml stock solution and keep in small aliquots (do not use once-thawed tubes)
D. Tween-20 (Sigma)
E. Yeast RNA (Sigma R6750)
F. Formamide (Sigma F7503), deionized with mixed bed resin TMD-8 (Sigma M8157)
G. DEPC-treated 20x SSC
H. 5M NaCl
I. 0.5M EDTA
J. Heparin (Sigma)
K. 1M Tris pH7.5
L. 2M Tris pH9.5
M. Sheep serum
N. Anti-DIG antibody, alkali-phosphatase conjugated (Roche)
O. Levamisole (Sigma L9756)
P. BCIP (Biosynth B7500) make 50mg/ml solution in
Q. NBT (Biosynth N8100)
NBT: 100 mg/ml in 70% dimethylformamide+30%H2O

BCIP: 50 mg/ml in dimethylformamide
Biosynth (http://www.biosynth.com/ASPUSA/index.asp) is a good source to buy powders of NBT (#N8100) and BCIP(#B7500).