# Manual non-radioactive RNA *in situ* hybridization steps with amplification strategy

Tissue: fresh frozen E14.5 embryo and adult brain sections (PFA-fixed, acetylated and dehydrated)

**Day 1**

| | | | | |
|---|---|---|---|---|
| *Incubate* | | Methanol + 3% H2O2 | 20 min | RT |
| *Wash* | | PBS | 5 min | RT |
| *Denature* | 0.2 N | HCl | 10 min | RT |
| *Wash* | | PBS | 2 x 5 min | RT |
| *Deproteinate* | | PK-buffer + 5-10 µg/ml proteinase K[1] | 2 x 10 min | RT |
| *Wash* | | PBS | 2 x 5 min | RT |
| *Fix* | 4 % | PFA in PBS | 15 min | RT |
| *Wash* | | PBS | 2 x 5 min | RT |
| *Pre-hybridize* | | HyB-Buffer (add 1mg/ml DTT) | 30 min | 60 °C |
| *Hybridize* | | HyB-Buffer + 0.5-2µg/ml probe (parafilmed) | o/n | 60 °C |

**Day 2**

| | | | | |
|---|---|---|---|---|
| *Wash* | 2x | SSC | 15 min | 60 °C |
| *Wash* | 2x | SSC | 5 min | RT |
| *Wash* | 0.2x | SSC | 2 x 30 min | 60 °C |
| *Wash* | 0.2x | SSC | 2 min | RT |
| *Wash* | | TNT buffer (0.1% Tween 20 in TN) | 2 x 20 min | RT |
| *Block* | 10 % | inactivated sheep serum (SS) in TNB | 30 min | RT |
| *Antibody* | 10 % | SS in TNB + anti-DIG (POD) antibody (1:500) | 30 min | RT |
| *Wash* | | TNT buffer (0.1% Tween 20 in TN) | 2 x 20 min | RT |
| *Incubate* | Tyramide Biotin (1:50 conc.) | | 20 min | RT |
| *Wash* | Maleate wash buffer | | 2 x 20 min | RT |
| *Incubate* | Neutravidin-AP | | 30 min | RT |
| *Wash* | Maleate wash buffer | | 2 x 20 min | RT |
| *Wash* | | TMN (pH 9.5; add 5mM Levamisole freshly) | 2 x 5 min | RT |
| *Stain* | | TMN + 1µl/ml NBT and 3.5µl/ml BCIP | 3 x 15 min[2] | RT dark |

**Day 3**

| | | | |
|---|---|---|---|
| *Wash* | PBS | 2 x 5 min | RT |
| Fix | 4 % PFA | 2 x 5 min | RT |
| *Wash* | PBS | 2 x 5 min | RT |
| *Mount* | Hydro-Mount (cover slipped) | > 120 min | 37 °C |

[1]The required proteinase K concentration depends largely on the tissue type. By default we use 5µl/100ml of proteinase K (Roche 3115828) for E14.5 mouse embryo and 35µl/100ml for adult mouse / monkey brain.

[2]Critical: Check the signal intensity every 10 mins during the last staining step. Higher expressed genes will be stained within 10 mins, whereas lower expressed genes might take 30 – 35 mins.