IN SITU HYBRIDIZATION PROTOCOL
LABELLING OF RNA PROBES BY IN VITRO TRANSCRIPTION

All reagents should be made up in DEPC-treated water

1. Add the following to a sterile microfuge tube at room temperature in order:

♦ 5 X transcription buffer	2 µl
♦ DTT	1 µl
♦ * Rnasin 20-40ug/ul	0.4 µl
♦ * Nucleotide mixture	2 µl
♦ Plasmid DNA (1 µg / µl)	1 µl
♦ Radiolabelled UTP (^{35}S) or CTP (^{32}P)	2.5 µl (25 µCi)
♦ RNA polymerase Mix on vortex and spinfor afew seconds up to 10.000 g.	1 µl (20 units)

2. Incubate the above mixture for a **minimum** of 1 hour at 37 °C.

3. To terminate the transcription, add 1 µl RNase-free DNase and incubate for 10 minutes at 37 °C to destroy the template.

4. **Separate** the probe from unincorporated nucleotides by adding the following:

♦ * tRNA 10 ug/ul	1 µl
♦ DEPC-treated water	184 µl
♦ 4M NaCl	5 µl
♦ Phenol/chloroform (1:1)	200 µl (is approx. equal volume of above mixture)

5. Mix, spin for 5 minutes at 12 000 *g* in a microfuge and collect the upper aqueous phase (+/-200 µl). Extract again with an equal volume of chloroform, then mix by vortexing and spin for 5 minutes at 12 000 *g*.

6. **Precipitation** : To second aquoeus phase add:
   - 7 M Ammonium Acetate 100 µl (2.5 M final conc.)
   - Absolute Ethanol (kept at - 20 C) 750 µl (approx. 2.5 vol.)

   vortex and spin to 10.000 g -leave to precipitate overnight at - 20 C or for 2 hours at - 80 C

7. Spin in a microfuge at 12 000 *g* for 25 minutes at 4 °C and discard the supernatant using a capilliary tube. Dry the RNA pellet under speed vacuum (approx 3 minutes, maximum 5 minutes) and, when dry, dissolve it in 20 µl DEPC-treated water. Remove 1 µl for assessment of incorporation of radioactivity.

8. Store ^{32}P-labelled probes at -20 °C and ^{35}S-labelled probes at -80 °C.

9. Count the radioactivity of the probe in a beta counter, using a standard scintillation fluid, and calculate the specific activity of the probe.

* t-Rna from Boehringer- Mannheim # 109541
* Rnasin -Promega # PR-N211-2500 U
* Riboprobe Nucleotide mixture Promega #P-1221 10mM each in 0.5 ml
Mix r.ATP,rCTP,rGTP 10ul of eachand 10 ul of Depc-H2O = 40 ul is stockmixture