RNA Probe Synthesis for *in situ* Hybridization

In addition to the reagents below, prepare sterile aliquots of the following: molecular grade water, 70% EtOH, and 100% EtOH.

Each reagent of the following reagents from the DIG RNA labeling Kit (SP6/T7) should be stored in the -20°C freezer:

**8** – Transcription Buffer, 10X

**7** – Nucleotide Mix (DIG), 10X

Gene-of-interest, Purified PCR fragment

 **10** – RNase Inhibitor

 **11** – SP6 RNA polymerase OR **12** – T7 RNA polymerase, (depending on fragment orientation)

**9** – DNase

1. Set thermocycler or heat block to incubate at 37°C. If using thermocycler, we recommend that the lid temperature is set to 105°C.
2. Place purified PCR reaction tube on ice and add the following:

 10.5 µl H2O for Probe Synthesis

 **8** – 2 µl Transcription Buffer, 10X

 **7** – 2 µl Nucleotide Mix (DIG), 10X

 For ‘SOX10’ – 4 µl Purified ‘SOX10’ PCR Fragment

 **10** – 0.5 µl RNase Inhibitor

 **11** – 1 µl SP6 RNA polymerase OR **12** – T7 RNA polymerase

 = Total volume of 20 µl in a 800 µL reaction tube.

1. Place PCR reaction tube in the thermocycler for 2 hours.
2. Add 1 µl DNase to the PCR tube. Thoroughly mix reaction, and spin down using a microfuge before returning to the thermocycler for another 15 minutes @ 37°C.
3. Add the following to an Eppendorf tube placed on ice:
4. 80 µl dH2O for Probe Synthesis
5. 10 µl 4m LiCl
6. 330 µl 100% EtOH
7. Using a p200, transfer the 21 µL total volume from the PCR reaction tube to the Eppendorf tube.
8. Vortex and centrifuge, then incubate at -20°C for at least 30 minutes, or overnight (for best results).
9. Balance and spin the Eppendorf tube in a refrigerated centrifuge (set to 4°C) for 10 minutes at 14,000K.
10. Decant liquid, but make certain to retain pellet. Check by simply tilting the Eppendorf tube with liquid in it while the cap is on.
11. Wash pellet with 200 µl 70% EtOH. Be certain to resuspend the pellet before placing the Eppendorf tube back into the centrifuge.
12. Place the Eppendorf tube in the refrigerated centrifuge (set to 4°C) for 10 minutes at 14,000K.
13. Repeat Steps 13 through 15.
14. **Do NOT decant the supernatant!** The pellet is most likely floating freely in the Eppendorf tube. Use a p200 pipet tip to pipette off ~200 µl of 70% EtOH.
15. Allow the pellet to dry for 10 minutes and dissolve the sample in 10 µl H2O.
16. Run 1 µl of the probe on a 1.5% gel at 70V for ~1.5 hours. We use ~5 µl of Marker VIII MW Ladder Roche) to estimate size and quantity of the synthesized probe.
17. Probe can now be used for whole-mount *in situ* hybridization.