In situ hybridization DIG RNA probe synthesis and clean up

Generation of PCR-based probe fragment

1. Follow standard primer design rules and generate a 500–1500 bp sequence-specific probe for your gene of interest.
    - Use blastn to compare the probe to refseq mRNA and ensure that no sequence contains >80% homology.
2. Add a the T7 promoter sequence plus an additional 3 – 7 bp* to the 5' end of the forward primer (for sense probe) and 5' end of the reverse primer (for antisense probe). You should have two primer pairs – one for the sense probe and one for the antisense probe:
    - Sense: forward primer (+T7), reverse primer (no T7)
    - Antisense: Forward primer (no T7), reverse primer (+T7)
    - *T7 polymerase does not bind well to the end of DNA. Adding an additional 3–7 bp improves binding efficiency to enhance transcription.
    - Example antisense primer pair (5' – 3'): extra-T7-primer
        - F: AAACCACCAACCGTCACAGA
        - R: cagtgaattgTAATACGACTCACTATAGGGCGTCCGGGCGATTGATACTA
3. Amplify probe fragment from cDNA, gel extract, and nanodrop to determine amplicon concentration.
4. Sequence probe.

T7 in vitro transcription

5. Setup transcription reaction for probe synthesis (all components can be found in the -20˚C):

Component	Volume (µL)
10X transcription reaction buffer (Ambion)	2
Roche 10X DIG NTP mix	2
PCR template (up to 1 ug)	-
T7 RNAP Plus* (20 U/µL); Ambion	2
Nuclease-free H_2O	To 20
Total	**20**

*contains RNase inhibitor

6. Incubate 37˚C for 2 hours.
7. Remove 1 µL for gel (pre-DNaseI sample).
8. Add 1 µL TURBO DNaseI (2 U/µL) (in vitro transcription box) and incubate 37˚ for 30 min.
9. Add 0.5 µL 0.5 M EDTA (pH 8) to inhibit reaction.
10. Remove 1 µL for gel (post-DNaseI sample).

Lithium chloride/ethanol precipitation

11. Prepare a 0.1 M LiCl, 75% ethanol precipitation:

Component	Volume (µL)	Final Conc.
Transcription reaction	19.5	-
LiCl (7.5 M, 50 mM EDTA)	1.3	0.1 M
100% Ethanol	75	75%
RNase-free H2O	4.2	-
Total	**100**	

12. Incubate at -20˚C for 30 min – overnight.

13. Run pre- and post-DNaseI treatment aliquots on agarose gel in 1X TAE.
14. Precipitate RNA by spinning down at max speed for 15 min.
15. Discard supernatant and wash pellet with ice-cold 70% ethanol to remove residual salt and unincorporated nucleotides.
16. Spin down max speed for 5 min.
17. Remove supernatant and let pellet air dry for 5–10 min (do not over dry pellet).
18. Resuspend pellet in 20 μL nuclease-free water.
19. Nanodrop sample (Correct for presence of DIG label ~ conc. x 5/7 according to Monica).
    - A260/A280 ~ 2 for pure RNA
    - A260/A230 > 2
20. Dilute probe in Hauptmann's hybridization solution to 0.5 mg/mL and store at -20˚C (this is your stock solution).

Materials

Hauptmann's hybridization buffer (50% formamide, 5x SSC, 50 ug/mL Heparin, 500 ug/mL yeast RNA). Prepare a 49.5 mL buffer without yeast RNA: Thaw 25 mL 100% formamide aliquot and add 12.5 mL 20x SSC, 50 μL Heparin (50 mg/mL), 50 μL Tween-20, and pH to 6.5 with 1M citric acid. Top up to 49.5 mL with H_2O. Make 9.9 mL aliquots in 15 mL falcon tubes. Take one aliquot and add 100 μL yeast RNA (50 mg/mL). The rest can be stored for future use.