8/20/99 K. Fitch

RNA probe synthesis:
(From K. Weiler)

(RNA probes are better for testis in-situ hybridization due to less background and higher specificity.)

1. Prepare probe DNA so that it is linearized and cut so that T3 or T7 RNA polymerase will transcribe the correct strand, i.e. the strand complementary to the sense RNA.

2. Transcription reaction:
Reaction components: (All RNAse free of course!)
2ug linearized DNA
4ul 10X transcription buffer (400mM Tris pH8, 60mM MgCl2, 100mM DDT, 20mM spermidine).
4ul Digogixenin-UTP NTP mix (from Boehringer Manheim)
2ul RNAsin (Promega 36U/ul)
1ul T7 polymerase (NEB 50U/ul) OR 4ul T3 polymerase (NEB 20u/ul).
H20 up to 40ul volume
Total volume = 40ul (This can be scaled down if necessary)

3. Incubate at 37(C for 2 hours, pulse spin, then place on ice.

4. Add 10ul H2O, + 2ul .5M EDTA, pH8 to stop reaction.

5. Hydrolyze transcripts by adding 50ul 2X carbonate buffer (80mM NaHCO3, 120mM Na2CO3, pH 10.2) and incubating at 60(C for approximately 40minutes, according to the following calculation:
time = (LI) initial fragment size ñ (LF)final fragment size
(.11kb/min) (LI) (LF)

desire~ 150 bp average = 57.6 minutes for a 3 kb transcript.

6. Neutralize reaction by adding 100ul of 0.2M NaOAc, pH 6.0, + 1ul glacial acetic acid.

7. To precipitate RNA, add 1ul 20mg/ml glycogen
20ul LiCl
600ul EtOH

freeze > 15 minutes at -20(C, spin 20 minutes at 4(C, wash with 70% EtOH and dry.

8. Resuspend pellet in 300ul H2O, aliquot, and store at -70(C. Should be stable for months. Use 10ul per 500uls hybridization solution.

Analysis of RNA Transcripts by Gel Electrophoresis

Prepare 1% agarose formaldehyde gel
0.5g agarose
+36mls H2O
microwave to dissolve, and cool to 60(C
+5ml 10X MOPS buffer @60(C.
+ 9mls 37% formaldehyde pH >4. (final conc. 6.7%)
Pour in hood.

Use 1X MOPS for running buffer.

Prepare denaturation solution for 2.5ul RNA samples + RNA ladder (prepare fresh).

Per sample:	final [ ]
0.9ul H2O	
10ul dI formamide	50%
3.5ul 37% formaldehyde	6.7%
2ul 10X MOPS	1X
1ul 10mg/ml EtBr	.5ug/ul

Add 17.5ul denaturation solution to 2.5ul RNA. Denature the samples at 55(C for 15minutes: immediately transfer to ice.
Add 2ul 10X loading buffer. Load gel. Run at ~50V for 3hrs.

10X loading buffer
5mls H2O
5mls glycerol
25mg Bromophenol blue
25mg Xylene Cyanol
20ul 0.5M EDTA, pH 8.

Final volume = 10mls

Testis in situ hybridization

We do these steps in small, reusable plastic ìbasketsî that fit in 24 well plate: Everything must be Rnase free, use DEPC treated water, etcÖ. Volumes are 1ml.

1. Dissect young males in 0.7% NaCl, store in 0.7% NaCl, on ice.

2. Fix for 20 min. to 1hr in 4% paraformaldehyde in Hepes buffer (0.1M Hepes pH 6.9, 2mM MsSO4, 1mM EGTA). at room temp for steps 2-9

3. Wash 3 X 5 min. in PBST (1XPBS + 0.1% Tween 20).

4. Incubate in 50ug/ml proteinase K 5-7 min (donít let go too long).

5. Stop reaction with 2mg/ml glycine for 2 min.

6. Wash 2X 5 min. in PBST.

7. Fix 20 min. in 4% paraformaldehyde in Hepes buffer.

8. Wash 3 X 10 minutes in PBST.

9. Wash 10 min in 1:1 ratio of PBST : Hybridization buffer.

10. Prehyb at least 1 hr at 65 C (for RNA probes) in Hyb.Buffer (preheat hyb buffer).

11. Heat denature the probe at 70C for 10 minutes, quench on ice. Heat hyb buffer to 65C and mix with probe (10ul probe in 500ul hyb buffer), add to tissue.

12. Hybridize overnight at 65C in a moist chamber. Does not need to shake.

13. Wash 6X 30 minutes in hyb buffer at 65C (at least 3hrs and 6 changes).

14. Wash 15-20 minutes in 4:1 hyb buffer:PBST at room temp. Repeat with 3:2, 2:3, 1:4 ratioís of hyb buffer to PBST.

15. Wash 2X 15 min. in PBST.

16. Incubate overnight in 1:2000 dilution of alkaline phosphatase conjugated anti-DIG antibody (from Boeringer Manheim) in 500ul PBST at 4C.

17. Wash 4 X 20 minutes in PBST.

18. Wash 3 X 5 min. NMTT (100mM NaCl, 50mM MgCl2, 100mM Tris, pH 9.5, 0.1% Tween 20 prepare fresh,).

19. Color reaction: 5min-1hr (4.5ul NBT, 3.5ul X-phosphate (both from Boeringer Manheim) in 1ml NMTT).

20. Stop reaction with PBST when it looks done (based on visualization under the microscope).

21. Incubate in 50% glycerol >1hr, 70% glycerol, >1hr., then overnight in 90% glycerol at 4C. Mount in 90% glycerol.

Solutions:

Hepes buffer
- 0.1M Hepes pH 6.9
- 2mM MsSO4
- 1mM EGTA

PBST
PBS + 0.1% Tween 20

Hybridization Buffer
- 50% deionized formamide
- 5 X SSC
- 100ug/ml sonicated salmon sperm DNA
- 50ug/ml Heparin
- 0.1% Tween 20

NMTT
100mM NaCl
- 50mM MgCl2
- 100mM Tris, pH 9.5
- 0.1% Tween 20