Alpers Lab

In Situ Hybridization

Use RNase free glassware, containers, slide carriers and solutions. Use RNaseZap to rinse containers, etc and ddH2O (or DEPC treated dH2O). Wear gloves!

Solutions - Day 1 (RNase free)

0.5x SSC
Proteinase K
Proteinase K buffer
Prehybridization solution
Box buffer

Solutions - Day 2

2x SSC
RNase A
RNase buffer
0.1x SSC + 0.5% Tween 20

DAY ONE

Use only green labelled (RNase free) solutions, glassware, etc.

1. Warm 200 ml Proteinase K buffer in 37°C water bath. Thaw proteinase K aliquot.

2. Label all slides with specimen number, probe and number of days to be exposed (or #1 and #2)

3. Deparaffinize:

Xylene x 3	10 min. each
100% Ethanol x 3	1-2 min. each
95% Ethanol x 2	1-2 min. each
70% Ethanol x 1	1-2 min. each
0.5x SSC rinse x 3	10 secs. each
RNase buffer rinse	10 secs. each

Use clean xylenes, alcohols

4. Add 100 ul stock proteinase K into 200 ml Proteinase K buffer just prior to adding slides. Incubate slides at 37°C for 30 minutes. (Note: the concentration of proteinase K can be varied to optimize hybridization - depends on type of tissue being used, but 5 ug/ml works well for kidney)

5. Wash 0.5xSSC x 3 30 secs each

Optional - Dehydrate slides using graded ethanols and allow to air dry

6. Make sure oven is warmed to 50°C. Lightly saturate paper lining the incubation boxes with box buffer. Boil the prehybe DTT solution for 5 minutes.

7. Blot the tissue sections on filter paper (not necessary if dehydrated and air dried), add 100 ul of prehyb DTT to the tissue. Place in incubation boxes and cover tightly.

8. Incubate for 2 hours at 50°C.

9. Determine the amount of probe needed - plan for 50 ul of hybridization solution per slide (make extra as solution is very viscous). Add approximately 500,000 cpm per slide.

   (50 ul) * (# of slides) = ul prehyb. solution needed

   500,000/(cpm probe) = ul probe per slide

   (ul probe per slide) * (# of slides) = ul probe to add to hyb. solution

10. Transfer the volume of probe needed to a microcentrifuge tube and denature the probe by placing in a 95°C water bath for 3 minutes.

11. Place the probe in an ice bath.

12. Mix the required amount of prehybridization solution with the probe. Mix well. This is the **hybridization solution.**

13. After the slides have been prehybridized for 2 hours, add 50 ul of the hybridization solution directly to the slides.

14. Incubate overnight at 50°C in humidity boxes.

DAY TWO

15. Transfer the slides in order to slide carriers and wash:

    2x SSC x 3 — 30 sec. each

    Discard first wash into radioactive waste container

16. Add 400 ul RNase A to 200 ml RNase buffer. Incubate slides for 30 minutes at 37°C.

17. Wash: 2x SSC x 3 — 30 sec. each

18. Wash: 0.1x SSC + 0.5% Tween x 3 — 40 min. each at 37 - 50°C*

    *Wash temperature determines stringency, higher temperature is more stringent. Need to determine optimal wash temp for each probe.

19. Wash: 2x SSC x 3 — 30 sec. each

20. Dehydrate:

50% EtOH + 0.3M Ammonium Acetate	2 min
70% EtOH + 0.3M Ammonium Acetate	2 min
90% EtOH + 0.3M Ammonium Acetate	2 min
100% EtOH x 2	2 min

21. Allow slides to air dry

22. Dip slides in emulsion (Kodak NTB2 diluted 1:1 with dH2O). After drying in dark room, place slides in slide boxes, seal the boxes with electrical tape and wrap in aluminum foil.

23. Allow slides to expose for 7 days to several months (depends on probe) in refrigerator. We usually develop one set of slides after 7 to 10 days, and then decide how long to let the second set of slides expose.

24. To develop - incubate the slides in Kodak D-12 developer for 5 minutes at 12°C, rinse in dH2O and then incubate in Kodak rapid fix (also at 12°C) for 5 minutes. After developing, the slides are washed in dH2O and then stained with hematoxylin and eosin, then coverslipped.