

***in situ* hybridization using digoxigenin-labeled probes and paraffin-embedded slides**

Before Day 1:

1. Make sure *in situ* jars and lids are autoclaved
2. Make sure there is plenty of DEPC water
3. Check availability of all necessary reagents
4. Choose proper paraffin-embedded slides
5. Make sure dig-riboprobes, substrate, and antibody are available.

Day 1:

1. All steps are carried out at room temperature unless otherwise stated.
2. Reserve one incubator at 68°C (needed O/N) and one at 60°C (morning)
3. Make 4% PFA (we make 4 times for a total of 200 ml)

4% PFA (200ml)

2 g PFA (hood)

6 ul 10 N NaOH (RNA use only)

~25 DEPC water

Stir with heat until 60°C (we make in 60°C incubator with rocking)

Cool on ice

Add 5 ml 10X PBS (RNA use only)

Add DEPC water to 50 ml.

Store unused solution in 15-50 ml Falcon tube at -20°C

4. Change all histology solutions in hood. Each green tub holds 250 ml.
5. Put slides in gray slide holder (sprayed with RNase Away and rinsed with DEPC water) and placed in 60°C incubator for 15 minutes.
6. Deparaffinize in 2 changes of xylene (2 minutes each)
7. Rehydration:

100% EtOH . . . 2 minutes

100% EtOH . . . 2 minutes

95% EtOH . . . 1 minute

95% EtOH . . . 1 minute

80% EtOH . . . 1 minute

70% EtOH . . . 1 minute

8. Rinse slides in *in situ* jar filled with DEPC water (200 ml)
9. Fix slides in 4% PFA for 10 minutes. SAVE PFA.
10. Wash slides 3X 3 minutes in 1X PBS

1X PBS (500 ml)

50 ml 10X Invitrogen RNA only PBS

DEPC water to 500 ml

11. Treat with Proteinase K solution for 4 minutes at room temperature

Proteinase K solution (200 ml) . . .

10 ml 1M Tris-HCl pH 7.5 (RNA only)

2 ml 0.5 M EDTA (RNA only)

400 ul Proteinase K solution (stored at -20°C, made with non-DEPC water to 10 mg/ml)

12. Post fix in 4% PFA for 5 minutes (use PFA from step #10.)
13. Rinse in 1X PBS (DEPC, RNA only) for 5 minutes
14. Incubate slides in TEA for 10 minutes to acetylate

TEA (200 ml): this MUST be made immediately before use!!

3.7 g Triethanolamine
448 ul 10 N NaOH (RNA only)
DEPC water to 200 ml
250 ul acetic anhydride

15. Wash slides 3X 5 minutes in 1X PBS (RNA only)
16. Place Whatman paper saturated with Saturation Buffer at the bottom of a plastic slide box. (3-5 layers of Whatman paper cut to 64mm x 205 mm.) Save excess Saturation Buffer for later.

Saturation Buffer (50 ml)

12.5 ml 20X SSC (with DEPC water)
25 ml 100% formamide (hood, stored in cold room)
12.5 ml DEPC water

17. Remove slides from PBS (one by one) and wipe the back and carefully around the tissues on front with a Kimwipe
18. Add ~50-200 ul (depending on tissue size) prehybridization buffer to each slide (covering tissue) and rest slides in slide box horizontally on top of slits

Prehybridization Solution (10 ml) *must thaw reagents

5 ml 100% Formamide (hood, stored in cold room)
2.5 ml 20X SSC pH 7.0 (DEPC, RNA only)
1 ml *50X Denhardt's Solution (-20°C, *in situ* box)
250 ul 10 mg/ml *yeast tRNA (-20°, *in situ* box)
500 ul 10 mg/ml *herring sperm DNA (-20°, *in situ* box)
750 ul DEPC water

19. Close slide box and prehybridize for 2-6 hours
20. Aliquot probe volume from -80°C stock, heat to 80°C (7 minutes), chill on ice briefly, and add to appropriate volume of prehybridization solution for final concentration of 0.3ng/ul.
21. Apply 50-200 ul probe in prehyb to each slide.
22. Cover each slice with a piece of parafilm cut to cover the tissue piece.
23. Add more saturation buffer to the Whatman paper in the slide box.
24. Close and tape up the slide box; carefully place the box in 68°C incubator overnight. Place a beaker of water in the incubator next to the slide box.

Day 2: (DEPC reagents no longer required)

1. Place slide holder filled with 0.2X SSC in water bath; heat to 55°C.

0.2X SSC (500 ml)

5.0 ml 20X SSC (non-DEPC)
495 ml ddH₂O

3. Incubate slides in 0.2X SSC for 1 hour @ 55°C.
4. Wash slides in 0.2X SSC @ room temperature for 5 minutes

5. Wash slides in Buffer B1 @ room temperature for 5 minutes

Buffer B1 (250 ml)

2.9 g Maleic Acid
2.175 g NaCl
ddH₂O to 225 ml
pH to 7.5 with 4.75 ml 10N NaOH
ddH₂O to 250 ml

6. Remove Whatman paper from slide box; replace with new Whatman paper (3-5 layers per side, 64mm x 205mm) and saturate with ddH₂O
7. Remove Buffer B1 from slides (one by one) with a Kimwipe
8. Make 1% blocking solution in Buffer B1 and use enough on slides to cover tissue (~200 ul). Block for 1 hour at room temperature.

1% Blocking Solution

500 ul 10% blocking solution (made in Buffer B1 from commercial blocking powder)
4.5 ml Buffer B1

9. Dump off blocking solution (one slide at a time) and wipe excess solution with a Kimwipe.
10. Dilute anti-digoxigenin antibody (BMB #1093274) to 1:1500 and add 200 ul or less to each slide. Cover with custom-cut pieces of parafilm. Close slide box lid and incubate for 2 hours at room temperature.

1:1500 dilution of anti-digoxigenin antibody

2.0 ul antibody (stored @ 4°C)
“Anti-Digoxigenin-AP Fab fragments”
3 ml Buffer B1

11. Rinse slides in Buffer B1 for 30 minutes. Repeat with another 30 minute rinse
12. Add 0.048 g levamisole to 200 ml of Buffer B3

Buffer B3 (200 ml)

10 ml 2M Tris pH 9.5
4 ml 5M NaCl
10 ml 1M MgCl₂
176 ml H₂O

13. Equilibrate slides in remaining Buffer B3/levamisole solution for 2-5 minutes
14. If necessary, add more water to Whatman paper in slide box.
15. Remove 5 ml Buffer B3/levamisole solution in a foil-wrapped 15 ml conical tube and add 17.5 ul NBT (BMB) to this aliquot; mix; add 17.5 ul BCIP (BMB). (Both reagents stored at -20°C).
16. Add approximately 100 ul to each tissue.
17. Tape box with lab tape and incubate 30 minutes-overnight at room temperature.

Day 3:

1. Carefully untape slide box and check slides under microscope for staining.
2. If no color, leave slides in closed box longer; a few more hours up to overnight.
3. To stop color reaction, wash slides in 1X PBS, 2 x 5 minutes
4. Mount coverslips by organic method:

70% Ethanol	1 min
80% Ethanol	1 min
95% Ethanol	1 min
95% Ethanol	1 min

100% Ethanol 1 min
100% Ethanol 1 min
Xylenes 2 min
Xylenes 2 min

Leave in Xylene until coverslip. Dry one by one and mount coverslip with organic mounting medium. Alternatively, slides can be mounted with aqueous medium without dehydration, but doing so will cause higher background levels.