

In-Situ Hybridization with DIG-probes on paraffin sections

A. Digoxigenin-labelled RNA probe:

DdH ₂ O	11.5 µl
10× Transcription Buffer	2.0 µl
0.1 M DTT	2.0 µl
Nucleotide mix (B-M)	2.0 µl
Linearized plasmid (1 µg/µl)	1.0 µl
Rnasin	0.5 µl
Polymerase	1.0 µl
Total Volume:	20.0 µl

37°C 2hr.

remove 1.0 µl, run on 1% Agarose gel

add 2.0 µl DNase I

37°C 15 minutes

add 100.0 µl TE, 10.0 µl 4M LiCl, 300.0 µl EtoH

-20°C 30 minutes

spin 10 minutes, 14K, remove supernatant

wash with 150.0 µl 70% EtoH, spin 2-5' 14K, remove supernatant

air dry pellet

resuspend in 100.0 µl TE

add 100.0 µl formamide

Store at -80°C

B. De-Wax slides:

1. Xylene 3×, 5 minutes each
2. 100% EtoH 2×, 5 minutes each
3. 90% EtoH 2 minutes
4. 70% EtoH 2 minutes
5. 40% EtoH 2 minutes
6. PBS 5 minutes
7. Proteinase K (40 µg/ml) in Pro. K. buffer for 7. Minutes at room temperature
8. PBS 5 minutes
9. Post-fix in 4% PFA in PBS 20 minutes
10. PBS 5 minutes
11. 2× SSC 5 minutes

12. Dehydrate quickly in 40% EtoH, 70% EtoH (leave at 70% for 10 minutes to remove any salt deposits), 90% EtoH, 100% EtoH, 100% EtoH.
13. Let Air dry until you are ready to put probe on but don't let the slides get dusty.

C. Hybridization:

1. Put 1.0 µg/ml of probe on each slide. Assume all probes are approximately 0.1µg/ul. Therefore, use 1.0 µl probe in 100.0 µl of hybridization mix for each slide. Place 50.0 µl on each half slide, spread a piece of parafilm through the probe drop to evenly cover the section, and slowly place the cover slip on top (try to avoid any bubbles).
2. Place slides in a moist chamber overnight at 70°C. Moist chambers: put a piece of whatmann paper and broken 5 ml pipets (at 2ml and 5.5ml) into the box, to form a platform for the slides. Pour enough moist chamber solution into the box to cover the bottom but don't fill it over the pipette base. Tape the box shut with yellow sticky tape.
3. Place a beaker of ddH2O in the hybridization oven with the moist chambers to keep the humidity level stable. This will prevent the moist chamber solution from evaporating over night.

D. Post-Hybridization Washes:

1. Wash in 50% Formamide/ 1× SSC/ 0.1% Tween 20 for 15 minutes at 65°C. Coverslips should fall off during this wash. If not, gently help them to fall off. Do not pull them off---this will destroy the tissue.
 2. Wash in the same solution 2×, 30 minutes each, 65°C
 3. Wash in 1× MABT twice, 30 minutes each R.T.
- *If background is very high for some probes, perform these additional steps after washing with 50% formamide/1×SSC/0.1% Tween 20 (step 2 above) but before washing with 1× MABT (step 3 above):*
 1. *Wash with STE (0.5 M NaCl, 10 mM Tris HCl pH 8.0, 5 mM EDTA) 10 minutes at R.T*
 2. *Wash an additional three times with STE, 10 minutes each, at 37°C*
 3. *RNase treatment: Add 10 µg/ml (200 µl 10 mg/ml RNase in 200 ml buffer) RNase A to STE buffer, 30 minutes, 37°C*
 4. *Wash with STE buffer at 37°C, 15 minutes.*

The RNase step does not increase the signal it just decreases the background with out affecting the signal.

E. Anti-Digoxigenin Immunohistochemistry:

1. Block slides in 500-800 μ l of MABT/ 2% BM Blocking reagent/ 10 % sheep serum in a humid chamber, without coverslips, at R.T, for 1.5 hours (This solution is tricky so follow the preparation directions carefully at the end of this protocol). Check the slides every 1/2 hour to be sure none of the sections are getting dry—add more solution if they are getting dry.
2. Add 1.0 μ l Anti-Dig antibody to 2 ml (1/2000 dilution) MABT/ 2% BM Blocking reagent/ 1% sheep serum. Place 100 μ l on each slide and coverslip with glass coverslips (try to avoid any bubbles).
3. Place in moist chamber overnight at R.T. Tape the box shut with yellow sticky tape.

F. Washes/ Detection:

1. Wash in 1 \times MABT + levamisole (0.048g/100ml) 4 to 5 \times , 30 minutes each at R.T. (Do not shake) The levamisole inhibits the endogenous A.P.
2. Wash in NTMT 5minutes to bring pH to 9.5 where A.P. is active
3. Place 500-800 μ l of BM purple on each slide and place in moist chamber. Cover the box with foil and leave at R.T. 4-24 hours. Anything longer will increase the background. You can place the chambers at 4 $^{\circ}$ C and the reaction will slow down considerably. When you remove them to R.T. again it will return to the original speed. Record the amount of time each probe requires for the optimal signal intensity. You may need to stop some of the slides before others by following the next five steps. However, you can re-use the 4% PFA for all of the slides whenever they are stopped. Place at -20 $^{\circ}$ C overnight, if this is necessary, and thaw at 55 $^{\circ}$ C for re-use.
4. Wash in PBS 5 minutes.
5. Refix in 4% PFA in PBS 20 minutes.
6. Wash in PBS 5 minutes. Remove slides and blot on a kimwipe tissue.
7. Mount in 80% Glycerol/PBS by adding 50 μ l to the blotted slide and coverslip.
8. Allow slides to dry overnight then seal with finger-nail polish.

G. Solution Preparation:

RNase FREE!!—prepare in sterile Tissue culture flasks (re-use the flask for alcohol's and 2× SSC but get a new flask for the Pro.K. solution.)

Proteinase K Solution: (200 ml)

10 ml 1M Tris pH 8.0

2 ml 0.5 M EDTA

320 µl 25mg/ml Pro. K. (Aliquot into 330 µl –do not freeze/thaw)

40% EtoH

120 ml ddH₂O

80 ml 100%EtoH

70% EtoH

60 ml ddH₂O

140 ml 100% EtoH

90% EtoH

11 ml ddH₂O

189 ml 95% EtoH

4% PFA

1.8 g in 45 ml PBS

Heat to 65°C shaking tubes periodically

2X SSC

20 ml 20X SSC

180 ml ddH₂O

Prepare in sterile conical tubes

Hybridization Mix

	Final concentration	For 10 ml	For 5 ml
10× salt	1 ×	1 ml	500 µl
Formamide	50 %	5 ml	2.5 ml
Dextran Sulfate	10 %	1 g	0.5 g
Yeast tRNA (10mg/ml, filtered)	1 mg/ml	1 ml	500 µl
100X Denhardt's	1 ×	100 µl	50 µl

ddH ₂ O		To 10 ml	To 5 ml
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Microwave the water and dextran to dissolve dextran (place conical tube in another flask with water to microwave). Then place at 70°C until you are ready to use—place it on ice and then bring to room temp. (This is to remove any bubbles that have formed).

10X Salt

	For 200 ml	For 100 ml	For 50 ml
NaCl	22.8 g	11.4 g	5.7 g
Tris Base	0.286 g	0.134 g	0.067 g
Tris HCL	2.8 g	1.4 g	0.7 g
NaH ₂ PO ₄	1.14 g	0.57 g	0.285 g
Na ₂ HPO ₄	1.42 g	0.71 g	0.355 g
0.5 M EDTA	5 ml	2.5 ml	1.25 ml
ddH ₂ O	To 200 ml	To 100 ml	To 50 ml

Moist Chamber Solution

	Final concentration	For 50 ml
Formamide	50 %	25 ml
20 × SSC	2 ×	5 ml
ddH ₂ O		20 ml

Post-Hyb. Washes

Reagents do need to be RNase Free!!!!

Formamide/SSC/Tween wash

	Final concentration	For 600 ml
Formamide	50 %	300 ml
20 × SSC	1 ×	30 ml
Tween 20	0.1 %	600 μl
ddH ₂ O		To 600 ml

5× MAB

	For 500 ml
Maleic Acid	29 g
NaCl	20.5 g

pH to 7.5 with approx. 30 ml 10 N NaOH or until solution becomes clear.

1× MABT

	For 500 ml
5 × MAB	100 ml
Tween 20	5 ml
ddH ₂ O	395 ml

Blocking Solution

	For 10 ml
MABT	10 ml
Blocking Reagent	0.2 g

*Microwave in large flask with saran wrap on top at power level 2 until dissolved. Do not let the solution boil –it will evaporate. Stop the microwave and then start it again. Continue this method until it is completely dissolved. It will be cloudy. Pour the solution into a conical tube. **If the volume is approximately 3 ml less than what you started with, you need to start over.** Cool this solution on ice and bring to room temp. before adding serum. Remove enough solution for the blocking step and add 10 % serum. The remaining solution will be used for diluting the antibody—add 1 % sheep serum to this and 1/2000 dilution of antibody.*

1× MABT + levamisole

0.048 g levamisole in 100 ml 1X MABT. Volume will depend on wash container:
0.096 g in 200 ml, 0.144 g in 300 ml, 0.193 g in 400 ml, 0.24 g in 500 ml etc...

NTMT

	For 600 ml	For 200 ml	For 100 ml
5 M NaCl	12 ml	4 ml	2 ml
2 M Tris, pH 9.5	30 ml	10 ml	5 ml
1 M MgCl ₂	30 ml	10 ml	5 ml
Tween 20	6 ml	2 ml	1 ml
ddH ₂ O	To 600 ml	To 200 ml	To 100 ml