IN SITU HYBRIDIZATION - FROZEN SECTIONS (From Nelly Polyak's Lab)

Collect 5-10 μM sections either on coated RNase-free or Superfrost/plus slides. If the tissue is prefixed you can air-dry slides for 1-2 hours @ RT before storage @ -20°C. For snap-frozen fresh tissue, freeze slides on dry ice immediately after cutting sections and store slides @ -80°C, eg. in RNase-free mailers or slide boxes.

A. Pretreatment of sections

1. Warm slides to RT and dry for 20 min @ 50°C

2. Place slides into mailers filled with 4% paraformaldehyde/PBS, pH 7-8. For prefixed tissue, fix 20 min @RT. For previously unfixed tissue it is good to fix longer (about an hour) @ +4°C. All day 1 treatments are done in RNase-free mailers.

3. Pour out fixative and wash slides in PBS 2 x 5 min @ RT. Always when pouring out liquids hold the slides back e.g. with a clean pipet tip and add the new buffer quickly so that the sections won't dry out.

4. Treat slides with 50 μg/ml proteinase K in PK buffer @ RT . Optimal time must be empirically determined. (For example, 1 min is good for snap-frozen breast tissue, 6 min for pre-fixed breast.)

5. Wash slides once in PBS for 5 min @ RT. Replace PBS with fresh fixative and fix slides in 4% paraformaldehyde/PBS for 15 min @ RT.

6. Rinse once in DepC-Water.

7. Treat slides with freshly prepared 0.1 M triethanolamine-HCl (pH 8.0) containing 0.25% acetic anhydride. Right before pouring out the water from the mailers, add acetic anhydride freshly into triethanolamine, mix well and pour into mailers. Incubate for 10 min @ RT.

8. Wash slides in PBS for 5 min @ RT

9. Transfer slides into mailers containing pre-warmed pre-hybridization solution. Pre-hybridize for 1-2 hour @ 55°C. Pre-hybe/hybe temperatures may need to be adjusted for optimal results with different probes).

10. Replace pre-hybe solution with same buffer into which 1 µg/ml of probe has been added (don't boil the probe, just pre-warm the whole solution to hybridization temperature) and continue incubation o/n (12-16 hours) @55°C.
You can collect pre-hybe solution to RNase free tube and store @ +4°C for a couple of months. When re-using it next time always add 1/3 fresh unused pre-hybe solution.

B. Washing steps & antibody visualization

1. Rinse slides in mailers 2 times with pre-warmed (same as hyb. temp.) 2X SSC. Transfer slides into a glass trough filled with pre-warmed 2X SSC and wash for 15 min @ hyb. temperature with gentle stirring.

2. Add 10 µg/ml RNase A into pre-warmed 2X SSC. Incubate slides for 30 min @ 37°C with gentle stirring. Rinse twice with 2X SSC @ RT.

3. Wash 2 x 30 min in 0.2X SSC @ hyb. temperature with gentle stirring or agitation.

4. Transfer slides to mailers. Wash 2 x 15 min in PBT @ RT.

5. Prepare antibody. Make 1:2000 dilution of antidigoxygenin antibody into PBT containing 1% heat inactivated & filtered goat serum. Can be prepared in advance during the blocking and let mix @ +4°C (not necessary to prepare in advance).

6. Block slides in PBT + 10% serum for 40-60 min @ RT.

7. Take slides out of the mailers one at the time. Shake well off the excess liquid and dry the borders with Kimwipe. Draw quickly lines with hydrophobic PAP pen on the long edges and the both ends of the slide. Place slides into a humidified chamber and add 500 µl of diluted antibody to each slide. Incubate o/n @ +4°C.

8. Pour off excess antibody solution and wash slides in PBT 3 x 30 min @ RT in mailers.

9. Wash slides in freshly prepared alkaline-phosphatase buffer 2 x 5 min @ RT (No levamisole in the first wash).

10. Color development. For every ml of fresh alkaline-phosphatase buffer add 1 µl of 75 mg/ml stock of NBT and 3.5 µl of 50 mg/ml stock of BCIP. Prepare this chromogen solution mixture right before use. Replace the wash buffer with the

chromogen solution and incubate in dark until desired signal intensity has been reached. Often 3-8 hours are needed for a strong signal. Make sure that the mailers used in these last steps (8-10) are new or very well cleaned after previous use or otherwise precipitation may occur which can lead into increased background.

11. Stop signal development by washing slides in PBS (+10 mM EDTA) 3 x 5 min and fix in 4% paraformaldehyde (+10mM EDTA) for a minimum of 20 min but even o/n @ +4°C is fine. Longer fixation time might help prevent accumulation of background staining if slides are stores for several months. If desired, counterstain in nuclear Fast Red (Vector H-3403) (Counterstain will faint away fairly quickly) and mount in aqueous mounting media (eg. Dako C0563).

BUFFERS & SOLUTIONS

PK buffer

50 mM Tris-HCL, pH 7.5
5 mM EDTA

0.1 M Triethanolamine, pH 8.0

4.17 g triethanolamine
225 ml ddH_2O
650 µl 50% Sodium hydroxide

For every 10 ml add 25 µl of Acetic Anhydride (final conc. 0.25%)

PBT

1 X PBS
2 mg/ml BSA

0.1% Triton X-100

Keeps good @ RT for 2-4 days, if stored for longer times starts becoming cloudy. Best if prepared separately each time.

Hybridization Solution

	for 50 ml:
50 % high-grade formamide	25 ml
5 X SSC	12.5 ml of 20 X SSC
0.3 mg/ml yeast tRNA	0.3 ml of 50 mg/ml in DepC-water
1 X Denhardt's	0.5 ml of 100X stock
0.1% Tween	0.5 ml of 10% Tween
5mM EDTA	0.5 ml of 0.5 M EDTA ph 8.0
	add to 50 ml with DepC-water

NBT

75 mg/ml Nitro Blue tetrazolium in 70% dimethylformamide and 30% water. Store @ -20, won't freeze.

BCIP

50 mg/ml 5-bromo 4-cloro-3 indolyl phosphate in 100% formamide. The BCIP salt which we have now is not soluble in formamide but only in water. Make 10mg/ml solution and add 5-fold amount in the chromagen step. Store @ -20 in aliquots since it'll freeze.

Alkaline-phosphatase buffer

100mM Tris, ph 9.5
50 mM $MgCl_2$
100 mM NaCl
0.1% Tween-20
(5 mM levamisole, which must be added fresh)

This buffer is always made fresh each time because Tris acidifies quickly in the presence of the other components.

IN SITU PROBE LABELING PROTOCOL JLD
5/9/03

Template for labeling: We use a PCR template (not the whole plasmid). Dilute the template to 20 ng/µl and use 25 µl of this dilution/reaction (= 500 ng DNA/reaction).

Mix for labeling reaction:

5.0 µl	10X Transcription buffer
4.5 µl	Enzyme (T7 or SP6 RNA polymerase)
0.5 µl	Rnasin
3.0 µl	Labeling mix
12 µl	ddH_2O
+ 25 µl DNA ⇒	50 µl total volume

- Incubate 2 h @37°C
- After 2 h, add 2 µl (20 Units) of Dnase, mix gently and incubate 10 min @ 37°C
- After 10 min, add another 2 µl of Dnase and incubate another 10 min @ 37°C
- Stop reaction by adding 52 µl of Stop buffer

Cleaning the probe: We use self-made columns but I'm sure you can also buy them ready –made (eg. Qiagen)

If using the method we use:

- Cut 1 ml pipet tip from the 1 ml mark and throw away the narrow tip. Take a 15 ml Falcon tube and insert the remaining top part of the tip into the bottom (this will work as a holder for the column during the centrifugation steps)
- Prepare the column by placing the QUIK-SEP micro-column on top of the cut pipet tip and adding there 500 µl of well mixed S300 sepharose slurry (containing 0.1% SDS)
- Spin 5 min, 2800 rpm
- Pick up the column and place it into RNase-free screw-cap eppendorf tube. Then drop this assembly into 15 ml Falcon on top of the pipet tip holder
- Add RNA probe carefully onto the sepharose bed. Spin 10 min, 2800 rpm (RT ok)
- Take the eppendorf tube containing the cleaned probe and precipitate RNA by adding 1/10 volume (=10 µl) 3 M NaOAc and 2.5 volumes (275 µl) pre-chilled 100% Ethanol; vortex and incubate 10 min @-80°C
- Spin down max speed for 20 min @ +4°C

- Discard the supernatant by pouring and wash the pellet with 500 µl of pre-chilled 70% Ethanol, keep tubes on ice at all times. Spin down for 3 min @ +4°C
- Discard the supernatant carefully (the pellet comes of easily from the bottom of the tube) and let the pellet dry for just a few minutes (on ice)
- Resuspend the probe in 50 µl Rnase-free water and store @ -80 °C
- Measure the concentration of the RNA-probe (Generally I get concentrations anywhere between 300 and 800 ng/µl)

When making the probes (at least for the first time), it's a good idea to run an aliquot of 1 µg of each on a 5% Acrylamide/8 M Urea gel to see that the probes are the expected size and that the concentration is close to what you would expect based on OD.

Additional information:

About the orientation: After cloning your gene of interest into the plasmid, make sure you know the orientation. Generally we prefere always the T7 polymerase to generate the antisense probe and SP6 polymerase to generate the sense, but it really doesn't matter as long as you can keep track which is which for each probe pair.

Stop buffer: 1% SDS
20 mM EDTA
20mM Tris, pH 7.5
100mM NaCl

Ordering Information:

Transcription buffer, 10X conc.	Boehringer Mannheim	1 465 384
DIG RNA Labeling Mix	Roche Diagnostics	1 277 073
RNasin RNase inhibitor	Promega	N211A
T7 RNA polymerase	Roche	881 775
SP6 RNA polymerase	Roche	1 487 671
DNase	Roche	776 785
QUIK-SEP Micro-column	PerkinElmer Wallac	QS-GSM
Sephacryl S-300 High Resolution	Amersham Pharmacia	17-0599-10