

***in situ* Hybridization for Tissue**

Frozen Sections:

1. Take chuck (plus tissues) out of cryostat (they should be at cryostat temperature). Quickly trim the block into a rectangle with a razor.
2. Place block back into the cryostat.
3. Place chuck into microtome head, or unembedded fresh frozen tissues are directly place to the microtome head with some fresh O.C.T.
4. Slice. Sections are mounted onto slides as they're sliced. Pick up the section(s) directly off the knife onto a polylysine coated slide. You don't have to touch the section, they will jump off the knife onto the slide.
 - 6 to 8 micron sections are fairly easily managed
 - the cryostat temperature should be about - 18°C.
5. Keep the slides with section(s) in the cryostat or on dry ice until just before using. The unfixed sections can be stored at - 70°C for up to six weeks. Air dry slides at least 20 minutes.

FIX:

4% paraformaldehyde	20' (5' if tissue is already fixed)
3X PBS	5'
1X PBS	5'
1X PBS	5'
30% EtOH	2'
60% EtOH	2'
80% EtOH	2'
95% EtOH	2'
100% EtOH	2'

note: make all paraformaldehyde and PBS solutions fresh each time, EtOH solutions can be reused at least 10 times. Section may be stored at room temperature for at least a month.

Pretreatment of slides for subsequent hybridization:

Pretreatment:

1. Incubate in 0.2N HCL at RT for 10'
2. Rinse in ddH2O for 30 seconds.
3. Place at 70°C in 2X SSC for 15'.
 - The SSC should be preheated before the slides are immersed in it and should remain at 70°C throughout the incubation.
4. Rinse in ddH2O.

Pronase Treatment:

1. Remove slides from rake and place them horizontally. With a Pastuer pipette, carefully drip the pronase solution onto the sections.

2. Incubate for 10' .
 - Pronase is at 0.25 mg/ml or appropriate concentration in pronase buffer.
 - The incubation time starts as soon as the first slide has been treated with pronase.
3. Pour off excess pronase and replace slides in slide rack.

Inhibit Pronase:

1. 2mg/ml glycine in 1X PBS 30''
2. 1X PBS 2 x 30''
3. Fix in 4% paraformaldehyde 5'
4. Rinse in 1X PBS 2 X 4'

Acetylate:

1. Place slides (in rack) into 500 mls of 0.1M Triethanolamine pH 8.0 (pH with HCL).
 - Slide rack should be raised of the bottom of the container so that a stir bar can spin under the rack. Plastic pipettes work well. Cut the pipette and place two pieces in the container. The slide rack can then rest on the pipette pieces.
2. Add, while stirring rapidly, 1.25 ml of acetic anhydride.
 - This should be added dropwise to ensure proper mixing.
 - Do the acetylation under the hood.
3. Incubate 10'. Stirring can be slowed down.
4. Rinse with 1X PBS 2 X 3'.

Dehydrate:

- | | |
|------------------|----|
| <u>30 % EtOH</u> | 2' |
| 60 % EtOH | 2' |
| 80 % EtOH | 2' |
| 95 % EtOH | 2' |
| 100% EtOH | 2' |

Air dry. Section can be used immediately or stored at RT for a couple of days.

Preparation of Riboprobes:

Final reaction conditions:

40mM Tris-HCL pH 8.0

6mM MgCl₂

4mM Spermidine

10mM DTT

100µg/ml BSA

0.1% Triton X-100

1U/µl RNAsin

500µM rNTPs

5 - 20µM 35 S-UTP

100µg/ml linearized DNA template

0.4U/µl enzyme (SP6 or T7 RNA polymerase)

Final reaction mixture:

10X reaction buffer (Tris, MgCl₂, Spermidine) 2µl

1mg/ml BSA 2µl

100mM DTT 2µl

1% Triton X-100 2µl

10mM ATP 1µl

10mM CTP 1µl

10mM GTP 1µl

RNAsin (40U/µl) 0.5µl

DNA template (1µg/µl) 2µl

35 S-UTP (resuspended vol.) 5.5µl

Sp6 (10U/µl) 1µl

Procedure:

1. Dry down label and resuspend it in an appropriate volume of ddH₂O.
2. Make the reaction mixture and incubate at 30°C for 2 - 4 hours.
3. Terminate reaction:
add 13µl of DNAase buffer (50mM Tris 7.5, 10mM MgCl₂), add 2µl of RNase free DNase (1U/µl), and incubate at 37°C for 30'.
4. Add 100 µl of TNE (100mM Tris 8.0, 0.15M NaCl, 5mM EDTA).
5. Determine the % incorporation:
Take 1µl of reaction mixture (step 6) to count (unincorp + incorp)
Take 1µl of reaction mixture (step 6) to TCA precipitate and count (incorporate).
6. Phenol/CHCl₃ extract the mixture (step 6).
7. CHCl₃ extract the mixture (step 6).
8. Add 10 - 50 µg or carrier RNA, an equal volume of 4M Na₄OAc. and 2.5 volumes of EtOH. Freeze.
9. Spin and dry.
10. Limited alkaline hydrolysis:

resuspend pellet in 80µl of 10mM DTT, add 20µl of 5X carbonate buffer pH 10.4. Incubate at 60°C for one hour. After hydrolysis, probe size should be 75 - 100 bp. Hydrolysis time is calculated from the formula:

$$t = \frac{L_o - L_f}{K \cdot L_o \cdot L_f}$$

L_o = initial fragment length
 L_f = final fragment length
 K = 0.11 Kb/min
 t = hydrolysis time (minutes)

11. Add 10µl 3M NaOAc and 2.5 volumes of EtOH. Freeze.
12. Spin, dry, and resuspend in an appropriate volume of hybridization buffer. The probe concentration is 0.15 - 0.3 ng/µl/Kb (where Kb is the length of the probe before hydrolysis). The amount of probe made is determined by the following formula:

probe (ng) = nmol label x fraction incorporated x 350ng/nmol X 4

nmol label = amount of label in reaction

fraction incorporated = amount of label incorporated

350 ng/nmol = average MW of NT

4 = factor to account for all NT's

Hybridizing the probe to the Tissue:

1. Place the probe on to the slides.
2. Put about 15µl of hybridization solution (HS) alongside of the tissue.
3. Let the HS move along the edge of the cover slip onto the tissue, avoid bubbles.
4. Gently and slowly lower the cover slip onto the tissue, avoid bubbles.
5. Seal the edge of the cover slip with rubber cement and let the cement dry.
6. Place the slides in a slide box (they don't need to be horizontal).
7. Place the slide box in a moist chamber (tupperware and wet paper towels). Make sure the water in the chamber will not evaporate.
8. Hybridize 12 - 18 hours at 55°C for riboprobes.

Washing the slides:

1. Remove cover slip from slides:
 - Gently peel off rubber cement
 - Stand slides in 2X SSC at room temperature for 5 minutes.
 - Gently pick up a slide, cover slip should fall off.
 - Dip slide a couple of times in the SSC.
2. Immediately transfer to a warmed 2XSSC, 10 mM DTT at 55°C for 30'.
3. Change to a fresh warmed 2XSSC, 10mM DTT at 55°C for 30'.

4. Incubate in 20 μ g/ml RNase A in 0.5M NaCl, 10mM Tris pH 8.0 at 37°C for 30'.
5. Rinse 3-5 times with an excess of 2XSSC.
6. Transfer to a fresh warmed 2XSSC, 10mM DTT, 50% formaldehyde at 55°C for 30'.
7. Change to a fresh warmed 2XSSC, 10mM DTT at 55°C for 30'.
8. Dehydrate slides through EtOH.
9. Air dry.

Putting the Slides on Emulsion:

1. Prepare Emulsion: Heat water bath to 45°C.
2. Put emulsion in water bath until melted (no longer than 10 - 15 minutes).
3. Slowly pour emulsion into a prewarmed dipper (45°C).
4. Dip slides one by one, keeping the emulsion at 45°C.
 - dip a slide, quickly wipe the emulsion off the back and place on a 4°C mental sheet (piece of mental lying on ice).
 - dip the slides as quickly as possible
5. Leave the slides on mental sheets for 5 -10 minutes.
6. Stand upright on slide drier and let them dry at RT for 8 hours to overnight.
7. Put slides into a light tight box and store desiccated at 4°C.
 - we use a small black slide box, seal the edges with tape and wrap it in three layers of aluminum foil.

Developing the slides:

1. Remove the slides from the refrigerator and let them come to RT.
2. Meanwhile, make up the fixer and the developer.
 - The developer is D-19 prepared as indicated on the package, but diluted 1:1.
 - The fixer is kodak fixer prepared as indicated on the package.
3. Chill the fixer, developer, and ddH₂O to 12 - 13°C.
 - this is so they will be at least 15°C when you want to use them.
4. Develop slides:
 - lights off (except safe light).
 - develop 4'
 - ddH₂O 10 "
 - fix 5'
 - light on

5. Place slides back in the ddH₂O and let them stand until they reach at least 20°C. Rinse at least 10 minutes in multiple changes of room temp. ddH₂O. This is to prevent the emulsion from cracking when they are stained.

Staining:

A. Giemsa Staining:

1. Stain slides in Giemsa:
 - stain slides for 3 - 5 ‘
 - different lots of of Giemsa seem to have different staining times, so test first before staining them all.
2. Dip slides into fresh ddH₂O and wipe off stain from the back of the slides.
3. Air dry.

B. Hematoxylin Staining:

1. Dip slides in stain for the optimum amount of time determined by staining an unimportant slide as a test.
2. **IMMEDIATELY** rinse the slides in **AT LEAST** three changes of ddH₂O for 10 seconds each.
3. Allow the slides to stand in the ddH₂O for two minutes.
4. Place slides into 0.1M ammonium hydroxide for 5 to 10 seconds and rinse once again in three fresh changes of ddH₂O. Allow the slides to stand in the last ddH₂O for two additional minutes.
5. Dehydrate the slides through ethanol and air dry.

The Final Step:

1. Mount each stained preparation under a cover slip with a small drop of Permunt
2. Alternatively, leave the autoradiographs uncovered and view them under immersion oil.
3. Use petroleum ether to remove oil from unmounted slides.
..... For all manipulations involving the emulsion, use a #2 SAFE LIGHT FILTER and a 15W bubble.

Preparations:

Coating the slides:

1. Wash slides
 - put slides into rack and let them sit in dilute HCL for 20 - 30’.
 - rinse with hot, running tap water for 0.5 - 1 hour.

- place in hot water and detergent for 1 -2 hours.
 - rinse with hot, running tap water 2 - 4 hours.
 - rinse with tap distilled water for 0.5 - 1 hour.
 - rinse 2 X 5' in ddH₂O
 - rinse 2 X 5' in 95% EtOH
 - air dry in a dust free environment.
 - *.* if using pre-cleaned slides, the above steps can be omitted.
2. Coat slides:
- dip slides into 50 -100 µg/ml polylysine in 10mM Tris - HCL 8.0 at RT for 10'.
 - air dry in a dust free environment. Slides may take up to 24 hours to dry.
3. Paraformaldehyde:
- all paraformaldehyde solution must be made up fresh in 1X PBS before use. It is good for about 12 hours after preparation.
 - To prepare: make up desired concentration in 1X PBS. Heat at about 70°C for about 30'. If it does not go into solution, add 1 -2 drops of 50% KOH. Make sure the pH of the solution does not go above 8.0.
 - cool paraformaldehyde to room temperature before use.
4. Pronase:
- autodigest a 40mg/ml stock solution at 37°C for 4 hours. make up solution in ddH₂O.
 - the pronase should be titrated each time a new stock is used.
- Make up a series of 2-fold dilution (0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg/ml) in pronase buffer and follow the pretreatment procedure until the fixation step. Instead of fixing, stain with Giemsa and examine the sections under the microscope. Determine the pronase concentration at which damage to the tissue is first detectable. Pretreat with a concentration two dilutions lower; e.g. if damage is first detectable at 1.0 mg/ml, use a concentration of 0.25 mg/ml for pretreatment.
5. Emulsion:
- dilute 1:1 with ddH₂O
 - prewarm water bath to 45°C.
 - place emulsion in water bath and let melt no longer than 30'.

- make sure the water level almost covers the bottle of emulsion, otherwise the emulsion will not melt quickly enough and background will be high.
- When emulsion is melted, mix it with an equal volume of 45°C ddH₂O gently. Rough handling increases background.
- aliquot (~ 15 mls) and store at 4°C in a light tight box. Do not freeze.

Solutions and Buffers:

5X Carbonate buffer:

200mM NaHCO₃

300mM Na₂CO₃

Geimsa:

1:20 dilution of stock in stock 10mM NaPO₄

1M NaPO₄:

138g NaH₂PO₄·H₂O

268g Na₂HPO₄· 7H₂O

ddH₂O to 2 liters

10X PBS:

1.3M NaCl

0.07M Na₂HPO₄

0.03M NaH₂PO₄

Pronase buffer:

50mM Tris-HCL 7.5

5mM EDTA, pH 8.0

20X SSC:

750g NaCl

353 g Sodium Citrate

ddH₂O to 4 liter

10 X transcription buffer:

400mM Tris-HCL pH 8.0

60mM MgCl₂

40mM Spermidine

RNA hybridization buffer:

50% Formamide (deionized)

10% dextran sulfate

0.3M NaCl

10mM Tris-HCL pH 8.0

10mM NaPO₄ pH 6.8

5mM EDTA

1X Denhardt's

10mM DTT

1mg/ml carrier RNA.

Chen Yan April 1989

Entered TAA 3-21-91

Updated by Ken September 1991