

**PROCEDURE FOR IN SITU HYBRIDIZATION OF FROZEN SECTIONS  
USING <sup>35</sup>S-LABELLED RIBOPROBES  
(from J. Histochem. Cytochem. 41, 1725-1733, 1993)**

1. Remove slides from freezer, thaw for 5min at 55°C.
2. Fix 10min in 4% paraformaldehyde 0.1M NaPO<sub>4</sub>, pH 7.4 at 4°C.
3. Wash 5min in 0.5xSSC (made in DEPC-H<sub>2</sub>O) at room temperature (RT)
4. Immerse slides in proteinase K solution , 1-5 µg/ml in RNase Buffer (made in DEPC-H<sub>2</sub>O) for 10min at RT. The amount of proteinase K needs to be optimized with each new preparation. Once optimized aliquots can be frozen down and used for some time.
5. Wash for 10min in 0.5xSSC (made in DEPC-H<sub>2</sub>O) at RT.
6. **PREHYBRIDIZATION:** Dry around sections with Kimwipe, lay slides flat in an air tight box with a piece of filter paper which has been saturated with Box Buffer (4xSSC, 50% formamide) on the bottom. Cover each section with 100µl of rHB2 without probe (can use 50µl if the tissue is small). Incubate at 42°C for 1-3 hours.
- 7a. **HYBRIDIZATION MIX:** for <sup>35</sup>S labeled riboprobe.  
Assuming that you have used 100µl of prehybridization buffer combine the following: 2.0µl probe per slide (stock solution 300,000 cpm/µl in 1XTE); 1.0 µl tRNA per slide (50 mg/ml stock in sdH<sub>2</sub>O) Heat 3min, 95°C immediately add 100µl ice cold rHB2 per slide, vortex, place on ice. (Adjust volumes if you have used less than 100ul for prehybridization).
- 7b. **HYBRIDIZATION:** Blot off most prehybridization solution from slides with small piece of filter paper. Add 100µl of above hybridization mix to each section. Incubate overnight at 55°C.
8. Wash 2x 10 minutes in 2xSSC with 10mM βME- 1mM EDTA at RT. (**HOT WASTE**)
9. Immerse in RNase A solution (20µg/ml in RNase buffer) 30 min at RT. (**HOT WASTE**)
10. Wash 2x 10min in 2xSSC with 10mM βME- 1mM EDTA at RT (**HOT WASTE**)
11. Wash 2 hours in 4 liters of 0.1xSSC with 10mM βME- 1mM EDTA, 55°C.
12. Wash 2x 10 minutes 0.5xSSC without βME or EDTA at RT.
13. Dehydrate 2min each in 50%, 70%, and 90% ethanol each containing 0.3M NH<sub>4</sub>Ac.
14. Dry in vacuum desiccator (3-4hrs), store with desiccant at RT until autoradiography.
15. Dip in Kodak NTB2 nuclear emulsion diluted 1:1 with water at 42°C, dry for 2 hours in the dark, expose in the dark at 4°C with desiccant for 2-12 weeks.
16. Develop at 15°C: a) 3min Kodak D19 diluted 1:1 with water  
b) 20 seconds in water stop  
c) 3min Kodak Fixer, full strength  
d) 3x 5min in water  
e) Counterstain with Hematoxylin and Eosin

## **<sup>35</sup>S-RIBOPROBE SYNTHESIS WITH PROBE SIZE REDUCTION AND PURIFICATION USING SPIN COLUMNS**

1. Pipet 12.5 $\mu$ l <sup>35</sup>S-UTP (1200 Ci/mmol) into eppendorf tube. Final concentration should be 12  $\mu$ M. Dry in speed vac. **Do not use nucleotide that has been previously thawed after receipt from manufacturer. Order 250  $\mu$ Ci vials and use them all up at once or throw the excess away.**

2. To tube with the dried down probe add:

2.0 $\mu$ l 5x transcription buffer  
1.0 $\mu$ l DTT, 100mM  
1.0 $\mu$ l RNasin  
1.0 $\mu$ l DNA (linearized plasmid 1 $\mu$ g/ $\mu$ l)  
2.0 $\mu$ l GTP+CTP+ATP Mix (stock sol.: 2.5 mM)  
2.0 $\mu$ l Sterile dH<sub>2</sub>O

Mix thoroughly and centrifuge

Add 1.0 $\mu$ l RNA Polymerase (SP6, T7 or T3 as required)

Mix gently by pipetting, do not vortex, incubate 1-2 hours, 37°C

(Order all of the above as ready-made stocks from Promega Biotech)

3. At the end of the incubation take 1  $\mu$ l of the transcription reaction, dilute 1/100 (add 99  $\mu$ l TE buffer) and keep it aside. This would be the before-column sample to be counted with an after-column sample to monitor the incorporation of <sup>35</sup>S-UTP (see steps 8 and 14).

4. Stop the reaction by adding:

1.0 $\mu$ l RQ1 DNase into the transcription reaction above

Mix well by pipetting

Incubate 15 minutes, 37°C

5. To extract RNA after DNase step, add:

20 $\mu$ l 1x TE

1.0 $\mu$ l tRNA (50 mg/ml)

Mix well by pipetting

6. To clean up the riboprobe:

Equilibrate one QuickSpin G-50 Sephadex column (Boehringer Mannheim) to room temperature for each riboprobe (15-30 min at room temperature)

Invert column gently for 20-25 times to suspend the gel

Remove **TOP** cap first, followed by the bottom cap

Allow the fluid within the column to drip through by gravity

Cut a collection tube (comes with column) ~5mm from bottom, place column in the collection tube and place the assembly in a 15ml tube

Spin for 2 min @ 1100g or 2500rpm (setting #6 on table top IEC centrifuge)

Discard fluid and collection tube

7. Place the column in a new collection tube, add riboprobe to the **CENTER** of the column. Place the assembly gently into the 15ml tube and spin for 4 min @ 1100g (#6 on the IEC). Remove the assembly gently with forceps, and measure the volume of riboprobe.

9. Take 3  $\mu\text{l}$  in a microfuge tube and keep it at  $-70\text{ }^{\circ}\text{C}$ . This sample will be used to verify the RNA transcripts on a 5% polyacrylamide gel (the next day, usually).

10. The rest of the riboprobe (30-40  $\mu\text{l}$ ) will be treated by alkaline hydrolysis to reduce its size as follows (Note that size reduction is only used on larger ( $300 < \text{bases}$ ) probes)

Add "x"  $\mu\text{l}$  TE buffer to adjust the volume to 50  $\mu\text{l}$

Add 50  $\mu\text{l}$  of 0.2 M  $\text{NaCO}_3$

Total volume: 100  $\mu\text{l}$

11. Incubate at  $60\text{ }^{\circ}\text{C}$  for t minutes, where t is defined by:

$$t = \frac{L_0 - L_f}{0.11 \times L_0 \times L_f} = \frac{0.7 \text{ Kb} - 0.2 \text{ Kb}}{0.11 \times 0.7 \times 0.2} = 32 \text{ min}$$

$L_0$  = Length of probe in Kb

$L_f$  = Final length of probe in Kb (Wilcox digested probe size is 0.2 Kb)

12. To stop the reaction, add:

6  $\mu\text{l}$  3 M NaAc pH 6.0

10  $\mu\text{l}$  10% glacial acetic acid

13. Precipitate the riboprobe, add:

22  $\mu\text{l}$  3 M NaAc, pH 6.0

600  $\mu\text{l}$  cold EtOH

2  $\mu\text{l}$  tRNA

Vortex, precipitate probe by placing it at  $-80\text{ }^{\circ}\text{C}$  (freezer or dry ice) for 2 hours. Precipitation can also be performed O/N at  $-80\text{ }^{\circ}\text{C}$ .

14. Spin down for 20 min in table top centrifuge in the cold room

16. Remove EtOH (**HOT WASTE**)

17. Wash pellet with 600  $\mu\text{l}$  cold EtOH and spin down as above for 20 min (**HOT WASTE**)

18. Speed Vac for 5 min to dry the RNA pellet

19. Redissolve pellet in 20-30  $\mu\text{l}$  TE buffer

20. Take 1  $\mu\text{l}$  of alkaline-digested riboprobe, count and adjust the total volume to a final concentration of 600,000 cpm/ $\mu\text{l}$  with TE buffer

21. From the adjusted volume keep 3  $\mu\text{l}$  to run a polyacrylamide gel

22. Make 50  $\mu\text{l}$  aliquots and store at  $-70\text{ }^{\circ}\text{C}$  up to one week. Use for in situ hybridization.

23. To determine the incorporation of  $^{35}\text{S}$ -UTP into the RNA transcripts:

Pipet 1.0  $\mu\text{l}$  of 1/100 dilution samples (end of transcription, before- and after-column) onto a small piece of Whatman DE81 paper.

Wash 3x 5min in 0.5M  $\text{NaPO}_4$ , pH 7.4

Rinse briefly (<10sec) in 100% EtOH  
Dry thoroughly and count in 10ml scintillation fluid

### **Verification of transcription**

1. Pour a 5.2% polyacrylamide urea gel
2. Thaw 3  $\mu$ l riboprobe samples kept from the previous day
3. Take 1  $\mu$ l, count and adjust to 300,000 cpm/ $\mu$ l
4. Load 600,000 cpm per probe in 1x SQB
5. Expose gel 12-24 hrs in a cassette with enhancing screens at  $-70^{\circ}\text{C}$

### **5.2% polyacrylamide gel**

4.2 g urea  
3.3 ml sdH<sub>2</sub>O  
1 ml 10x TBE  
1.3 ml 40% acrylamide  
40  $\mu$ l 10% APS  
20  $\mu$ l TEMED

## **TISSUE PREPARATION PROTOCOL FOR IN SITU HYBRIDIZATION ON FROZEN SECTIONS**

1. Remove tissue and rinse in PBS or saline
2. Immerse in 4% paraformaldehyde 0.1M sodium phosphate buffer pH7.4 at 4°C for 1-3hrs. Try to avoid overnight fixation if possible as this causes problems with keeping the section on the slide during the hybridization procedure.
3. Immerse in sterile 15% sucrose-PBS solution 3hrs-overnight at 4°C.
4. Embed tissue in O.C.T. (Baxter #M7148-4), M1 (Lipshaw) or any other convenient embedding matrix for frozen sectioning. Tissue should be oriented in the block appropriately for sectioning (cross-section etc.). Note the tissue number on the block directly and indicate which face of the block should be sectioned.
5. Freeze block with tissue in liquid nitrogen. Place approximately the bottom third of the block into the liquid nitrogen, allow to freeze until all but the center is frozen, allow freezing to continue on dry ice.
6. Store at -70°C in a sealed container or wrapped in foil and ship on dry ice.

It is also possible to use fresh frozen tissue for in situ hybridization if the paraformaldehyde/sucrose method is not feasible. Tissue should be rinsed in saline or PBS and frozen in liquid nitrogen in O.C.T. blocks as outlined above. Although not optimal it is also possible to use snap frozen material tissue without an embedding matrix. The fixation, sucrose, and O.C.T. steps are used primarily to improve the tissue morphology.

It is expected that the fixation times outlined above will not result in complete fixation of large pieces of tissue. However, the fixation step at the beginning of the hybridization procedure should ensure adequate fixation of such tissues prior to hybridization.

This protocol has been used successfully on large (up to 1cm<sup>3</sup>) and small (1mm<sup>3</sup>) tissue samples.

## **TISSUE PREPARATION PROTOCOL FOR IN SITU HYBRIDIZATION ON PARAFFIN SECTIONS**

1. Fix for 24 hrs in 4% paraformaldehyde
2. Process with conventional paraffin protocol (we use 1hr cycles 2x 70%EtOH, 2x 95% EtOH, 2x 100% EtOH, 2x Xylene, 2x paraffin)
3. Prepare blocks and section onto SuperFrost/Plus microscope slides

## **USE OF SUPERFROST/PLUS MICROSCOPE SLIDES**

We have used SuperFrost/Plus microscope slides for all of our frozen tissue sectioning for the past 9 years and have very good results as regards tissue retention on the slide after in situ hybridization. The advantage of using SuperFrost/Plus slides is they require no preparation time in the laboratory and are competitive in terms of cost when you consider technician time and reagent expenses.

## SECTIONING OF FROZEN TISSUE FOR IN SITU HYBRIDIZATION

Frozen tissues prepared as described can be wrapped and stored for many years prior to sectioning without loss of the mRNA signal. The biggest problem with stored tissue blocks is that they tend to desiccate if not wrapped properly and the OCT can be difficult to cut.

Blocks should be removed from the -70°C freezer and allowed to equilibrate with the cryostat chamber temperature. Tissues can be cut at any convenient temperature (-15 to -35°C) as needed. Most tissues cut well at -15°C (brain, kidney, liver, vessels, muscle, etc.) however fatty or more difficult tissues (adipose tissue, skin, lung) require temperatures as low as -35°C or more to obtain good sections. Vectabond coated slides should be kept at room temperature. Care should be taken not to touch the face of the slides but handle by the edges only. Frozen sections 5-7µm (thinner is OK but thicker, over 10µm, may present problems) should be cut, thaw-mounted on the room-temperature coated slides, and the slide with the section **immediately** refrozen by placing into a slide box (VWR micro slide box #48444-003) with a single desiccant capsule (Humi-Cap see below). When the box is full, place the top on the box and store at -70°C. Sections cut and stored with desiccant are stable for in situ hybridization and immunohistochemistry for most antigens for over 5 years.

### BUFFERS AND SOLUTIONS FOR IN SITU HYBRIDIZATION USING <sup>35</sup>S-LABELED RIBOPROBES ON FROZEN SECTIONS

<b>rHB2 (for riboprobes)</b>	<b>Stock</b>	<b>Volume</b>
10mM DTT	--	46.26mg
sdH <sub>2</sub> O	--	5.7ml
0.3M NaCl	5M	1.8ml
20mM TRIS, pH8.0	1M	600µl
5mMEDTA	250mM	600µl
1x Denhardt's	100x	300µl
10% Dextran Sulfate	50%	6.0ml
50% Formamide	100%	15.0ml
Makes 30ml		

<b>HB8 (for oligos)</b>	<b>Stock</b>	<b>Volume</b>
10mM DTT	--	46.26mg
sdH <sub>2</sub> O	--	9.84ml
1x Denhardt's	100x	300µl
5xSSC	20x	7.5ml
100µg/ml ssDNA	10mg/ml	300µl
100µg/ml tRNA	50mg/ml	60µl
10% Dextran Sulfate	50%	6.0ml
20% Formamide	100%	6.0ml
Makes 30ml		

<b>RNAse Buffer</b>	<b>Stock</b>	<b>Volume</b>
500mM NaCl	5M	100ml
10mM TRIS, pH8.0	1M	10ml
dH <sub>2</sub> O	--	890ml
Makes 1 liter		

<b>2xSSC, BME, EDTA</b>	<b>Stock</b>	<b>Volume</b>
2xSSC	20x	100ml
10mM $\beta$ -mercaptoethanol	--	875 $\mu$ l
1mM EDTA	250mM	4.0ml
dH <sub>2</sub> O	--	896ml

<b>Box Buffer</b>	<b>Stock</b>	<b>Volume</b>
4xSSC	20x	50ml
50% Formamide	100%	125ml
dH <sub>2</sub> O	--	75ml
Makes 250ml		

<b>Stringency Buffer</b>	<b>Stock</b>	<b>Volume</b>
0.1xSSC	20xSSC	20.0ml
10mM $\beta$ -mercaptoethanol		3.5ml
1mM EDTA	250mM	16.0ml
dH <sub>2</sub> O		4000ml
Makes 4 liters		

<b>Dehydration Buffers</b>	<b>50%</b>	<b>70%</b>	<b>90%</b>
100% EtOH	100ml	140ml	180ml
3M NH <sub>4</sub> Ac	20ml	20ml	20ml
dH <sub>2</sub> O	80ml	40ml	--

### GTP+CTP+ATP Mix for Transcription

5.0  $\mu$ l 10  $\mu$ M ATP  
 5.0  $\mu$ l 10  $\mu$ M CTP  
 5.0  $\mu$ l 10  $\mu$ M GTP  
 5.0  $\mu$ l sd H<sub>2</sub>O  
 20  $\mu$ l 2.5 mM NTP Mix

### RNase Stock (10mg/ml)

10mg RNase A (Sigma)  
 1.0ml RNase Buffer  
 Heat treat as per Maniatis 1st edition p.451

### Working RNase Solution-20 $\mu$ g/ml

300 $\mu$ l RNase Stock in 150ml RNase Buffer

### 50X Denhardt's

1g Ficoll  
 1g Polyvinylpyrrolidone  
 1g BSA  
 Add sterile distilled H<sub>2</sub>O to 100 ml

### Proteinase K

(20mg/ml) stock made up in water, frozen in aliquots which are used only once

#### **4% Paraformaldehyde**

Mix in a two liter flask:

200ml 0.5M NaPO<sub>4</sub>, pH 7.4

800ml sdH<sub>2</sub>O

Heat to 70°C with stirring on hot plate **IN HOOD**

Add 40g Paraformaldehyde (EM grade, Polysciences, cat# 0380)

**Note:** Once the solution has cleared (it should take 5 minutes or less) filter with a Whatman #2 filter. Immediately pour the solution into a one liter bottle which has been packed in ice. This is to cool the solution as quickly as possible to prevent breakdown of the paraformaldehyde. Store at 4°C for up to two weeks.

#### **15% Sucrose in PBS**

500ml sterile PBS

75g "RNase free" sucrose

Mix above and filter sterilize with Nalgene filtration unit type S(0.45 micron)

Store at 4°C

### **SOURCE OF MATERIALS USED FOR IN SITU HYBRIDIZATION (not meant as a recommendation but to aid in finding items needed)**

United Dessicants, 6845 Westfield Ave., Pennsauken, NJ. 08110-1582 USA (609-662-6500)  
Humi-Cap dessicant capsules (#245-2)

Baxter Scientific Products

Nalgene utility boxes (#L1995-4)

Miles stain dishes (#S7631-6)

Miles stain racks (#S7636)

O.C.T. (#M7148-4)

Tissue culture chamber slides (#T4135-4)

Peel-a-Way tissue molds (#M7275-3, #M7275-2)

Polysciences, Inc., 400 Valley Rd., Warrington, PA 18976 USA (800- 523-2575)

Gills hematoxylin #2 (#4570)

Alcoholic Eosin Y 1% (#17269)

VWR

Micro slide boxes black (holds 25@) (#48444-003)

Slide grips (#48440-002)

International Biotechnologies, Inc.(IBI), P.O. Box 9558, 275 Winchester Ave., New Haven, CT, 06535  
USA (800-243-2555)

Kodak NTB2 emulsion (#1654433)

Whatman

3MM paper (#3030M917)

DE81 filter paper (#3658M323)

Fisher Scientific

Autoimmerse heater (#L1995-4)

Superfrost/Plus Microscope Slides (#12-550-15)

Promega Corp., 2800 Woods Hollow Rd., Madison, WI 53711 USA (800-356-9526)  
Transcription buffer kits (ie. #P2490)  
RNasin (#N2511)

Amersham Corp., 2636 South Clearbrook Dr., Arlington Heights, IL 60005 USA  
(800-323-9750)  
35S-UTP (for riboprobe transcriptions) (#SJ1303 )  
35S-dATP (low DTT concentration for tailing rxions) (#SJ1334)

Sigma Chemical, P.O. Box 14508, St. Louis, MO 63178 USA (1-800-325-3010)  
RNase A (#R5125)  
Proteinase K (#P4914)  
tRNA (#R9001)

Vector Laboratories, Inc., 30 Ingold Road, Burlingame, CA 94010 USA (415-697-3600)  
Vectabond (#SP-1800)  
Vector Substrate kit I (#SK-5100)

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