McCONNELL LAB PROTOCOL FOR IN SITU HYBRIDIZATION USING RADIOACTIVE PROBE

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PREPARATION OF PROBES

^{35}S UTP (Dupont NEN 039H: 12.5mCi/ml)	8ul
0.1M DTT (0.01M DTT for SP6)	2ul
RNase Inhibitor (Gibco, human)	1ul
10mM rNTP (A,C,G no U)	2ul
5XT3/T7 buffer (or 5X SP6 buffer)	4ul
RNA polymerase (T3, T7 or SP6 polymerase)	1ul
linearized plasmid (0.05ug/ul)	2ul

Incubate 1 HR at 37ºC

Add 1unit DNAse (Roche RNase free, 10units/ul), incubate 15 MIN at 37ºC.
Stop reaction with 10ul 0.25M EDTA + 10ug glycogen, 100ul H_2O.
Add 50ul 6M Ammonium Acetate, 450ul 100% ETOH, freeze at -80ºC
Microfuge for 30MIN, decant, wash pellet w/ 80% ETOH, then wash w/ 100% ETOH.
Dry.

Resuspend pellet in100ul SET + DTT.
SET= 1% SDS in 10mM Tris, pH7.4, 1mM EDTA
1ml= 10ul Tris pH7.4
4ul 250 mM EDTA, DEPC treated
100ul 10% SDS
786ul DEPC treated H_2O
100ul 100mM DTT
This protocol can produce up to 200 million cpm, enough for 200 slides @ 5 million cpm/ml hybridization buffer. For larger scale screens, the prep is modified as follows:

probes generated from minipreps: 1/3 of plasmid DNA isolated from a 3ml culture is linearized, cleaned by phenol:chloroform extraction and precipitation with glycogen. Resuspend in 8ul DEPC treated H_2O.

For 30 RXNs:	
35S UTP	85ul
0.1M DTT (0.01M for SP6)	15ul
5X polymerase buffer	32ul
RNase inhibitor	7.5ul
10mM rNTP	15ul
RNA polymerase	7.5ul

Make up probe to 5 $X10^6$ cpm/ml final concentration in the following:

	2ml (10ml final)	1ml (5ml final)
E. coli tRNA (8mg/ml)	625ul	312.5ul
1M DTT	100ul	50ul
probe	$5X10^7$ cpm	$2.5X10^7$ cpm
DEPC H_2O	to 2ml	to 1ml

Mix and heat 5MIN 65ºC then add

Hybridization buffer	8ml	4ml

Hybridization buffer:	40ml	8ml	4ml
De ionized formamide	25ml	5ml	2.5ml
50% Dextran sulfate	10ml	2ml	1 ml
5M NaCl	3ml	0.6ml	0.3ml
100X Denhardt's	0.5ml	0.1ml	50ul
1M Tris, pH8.0	0.5ml	0.1ml	50ul
250mM EDTA, pH 8.0	0.2ml	40ul	20ul
DEPC treated H_2O	0.8ml	0.16ml	80ul

Vortex well and store at -80ºC until use (good for about 1 month).

TISSUE PREPARATION AND SECTIONING

Unfixed brains are removed and embedded in OCT medium, frozen on dry ice and stored at -80ºC until use. We have sucessfully used 3 year old frozen tissue. Formaldehyde fixed tissue has also been used sucessfully .

15 micron cryostat sections are thaw mounted onto treated slides (Superfrost from Fisher, most other brands work as well) and allowed to air dry. They are batch processed in slide boats through the following:

4% formaldehyde in PBS (fresh)	30 MIN
PBS	10 MIN X 3
50% ETOH	3 MIN
70% ETOH	3 MIN
95% ETOH	3 MIN X 2

Store in boxes at -80ºC with dessicant. We have used slides up to 2 months after sectioning.

TISSUE PRETREATMENTS

Bring slides in their boxes to room temp. Slides are batch processed in slide boats containing approximately 200ml solution.

Proteinase K digestion 30 MIN room temperature

	for 1000ml	for 800ml	for 600ml
1M Tris pH8.0, DEPC treated	93.75ml	75ml	56.25ml
500mM EDTA, pH8, DEPC	93.75ml	75ml	56.25ml
Distilled H_2O, not DEPC	812ml	650ml	487.5ml

For embryonic and neonatal brains, use 30-35ul proteinase K (10mg/ml predigested 30MIN) in each boat. For adult tissue, use 70-75ul proteinase K.

Rinse in distilled H2O 3 MIN

Rinse in 0.1M TEA pH8.0 3 MIN

Acetylation: 10 MIN
Add 500ul acetic anhydride to dry staining dishes,
put in slide trays and add 200ml 0.1M TEA.
Agitate vigorously.

Wash with 2X SSC 2 MIN X 2

Dehydrate through 50%/70%/95%/100%/100% ETOH 3 MIN ea

Air dry.

HYBRIDIZATION

Heat hyb mix at 65ºC for 10 MIN.
Add 180-200ul hyb mix per slide, spread and cover with parafilm coverslips. Place slides in small square petri dishes, place covered dishes on water soaked towels in plastic incubation boxes. Seal incubation boxes in plastic wrap, place in 60ºC incubator overnight.

Next Day:

Soak off coverslips in 4X SSC (200ml 20X SSC/1000ml). Forceps can be used to coax off recalcitrant coverslips.
Wash 2X 5 MIN in 4X SSC

RNASE DIGESTION 30 MIN @ 37ºC

RNase buffer	for 2000ml	for 1600ml
5M NaCl	200ml	160ml
1M Tris ph 8.0	20 ml	16ml
500mM EDTA	4ml	3.2ml
distilled H_2O		

Add 1ml 10mg/ml RNase A (previously boiled) to each 200ml boat. Agitate.

Washes:
2X 5 MIN room temp 2X SSC+ 1ul/ml DTT (100ml 20X SSC/L, 1ml 1M DTT)
1X10 MIN room temp 1X SSC + DTT (50ml 20X SSC/L, 1ml 1M DTT)
1X10 MIN room temp 0.5X SSC+DTT(25ml 20X SSC/L, 1ml 1M DTT)
1X30 MIN 60ºC 0.1X SSC+DTT (5ml 20X SSC/L, 1ml 1M DTT)
1X 5 MIN room temp 0.1M SSC+DTT

Dehydrate through 50%/70%/95%/100%/100%/100% ETOH 3 MIN ea

Air dry, place against film. Develop after 3 days room temperature.
Defat brain tissue through:
95%/100%/100% ETOH/ Xylene 5MIN
Xylene 30MIN
100% ETOH 3X 5 MIN
Air dry

Dip slides into Kodak NTB2 emulsion, diluted 1:1 with distilled H2O. Incubate in sealed boxes containing Drierite @ 4ºC.

Determine slide exposure from intensity of film image. Develop in Kodak D19 developer
2 MIN @ 16ºC, rinse in water, fix in Rapid Fix 4 MIN@ 16ºC. Wash slides 90 MIN in running water, stain with cresyl violet.

SOLUTIONS

Formaldehyde: make fresh, OK for a few days
for 800ML: heat 650ml H_2O to 60ºC. Add 32g paraformaldehyde, stirring, add 10 drops 10N NaOH until solution clears. Filter, cool to room temp, add 80ml 10X PBS, pH to 7.0, add water to final volume. Store @ 4ºC.

10X PBS : for 2000ml

151.9g NaCl
8.28g NaH_2PO_4-H_20
37.5g Na_2HPO_4-$7H_2O$

DEPC treat (see below) and autoclave if needed.

DEPC Treatment
1ml DEPC/liter. Shake up and let sit at least 1HR. Autoclave 1HR to inactivate DEPC.

1M DTT: use ultrapure DTT. Dissolve 15.4g DTT in 100ml 10mM sodium acetate buffer pH 5.2 (made in DEPC H_20) Store at -20ºC.

100X Denhardt's: 2%polyvinylpryrolidone (Gibco 130170), 2% ficoll (Sigma #F4375), 2% BSA (Sigma #A4503) mixed in DEPC water. Filter sterilize, store @-20ºC.

E. coli tRNA: (Roche 109541) 8 mg/ml in DEPC water. Store at –20ºC.

Proteinase K: 10mg/ml in 50mM Tris ph 8.0, 5mM EDTA. Predigest for 30 MIN at 37ºC, store @ -20ºC.

RNase A: 10mg/ml in 10mMTris pH 7.5, 15mMNaCl. Boil for 15 MIN, cool slowly to room temperature. Store @ -20ºC.

Tris solutions made with DEPC treated water.

TEA: 1M triethanolamine, pH to 8. No DEPC treatment, just distilled water.

20X SSC: for 1000ml

175.32g NaCl
88.23g sodium citrate-$2H_2O$

pH to 7, DEPC treat and autoclave if needed.